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RADIOACTIVE ISOTOPES IN BIOCHEMISTRY

by

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With a Preface by

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PREFACE

The use of isotopic tracers in biochemistry has grown from a very modest beginning to an immense structure in the course of 35 years. This development is quite remarkable, even in view of the fact that many branches of science have experienced extraordinary growth in the past few decades. The rapid development of biochemistry and the ready availability of labeled compounds have contributed largely to this result. The production of highly-active radioisotopes of most of the elements is made possible by the powerful beams of neutrons emerging from many present-day reactors. The chemist exhibits great skill in incorporating these radioactive tracers into a very large number of compounds—the catalog of Amersham (in England) alone contains 129 compounds labeled with ^{14}C.

The daily appearance of numerous biochemical studies employing radioactive tracers renders difficult the writing of a textbook on the use of radioisotopes. The author of the present book has found a happy solution to this problem. He adduces well-selected examples of problems which can only be solved by the use of radioactive tracers or whose solution is made appreciably easier by their use. Furthermore, he describes the techniques which are used in investigations with isotopic tracers. The fundamentals of radiochemistry, radiation chemistry, and radiation biology are also discussed.

When Wilhelm Ostwald was asked how he managed to turn out a large number of extensive volumes in a short span of years, he replied that his daughter stood at his left with blank sheets of paper, and his son at his right, taking away the finished sheets. In view of the fact that the author of the present work is now able to add to the books which have appeared in recent years this new and most successful one, one is tempted to believe that he has at his disposal some such system as that of Ostwald. He is to be congratulated on his performance.

G. DE HEVESY

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CHAPTER I

INTRODUCTION

1. Historical Survey

The importance of isotope methods in biology has frequently been compared with that of the microscope. Just as the invention of the microscope in the 17th century advanced the science of living tissues by tremendous strides, and made possible the later discovery of cells and microbes, so does the employment of isotopic methods put us in a position to investigate the details of metabolism in a reliable and highly sensitive manner.

The isotope—or tracer—technique originated at the Institut für Radiumforschung in Vienna, where G. Hevesy and F. Paneth carried out their classic researches shortly before the first World War. The first applications of the method were to the problems of inorganic and physical chemistry; solubilities of salts and rates of exchange of atoms between solids and solutions were determined. It seems astonishing in this era of speed that more than a decade elapsed before the new technique, so capable of producing results, was extended to a living system—once again, by Hevesy himself. The first biochemical application was an investigation of the uptake of lead by plants[1].

Even after the occurrence of the first biochemical investigation, progress was only halting. During the following years Hevesy and his coworkers published a few investigations on the metabolism of lead[2,3], bismuth[4] and thorium[3] in animals, and other authors, many of whom were directly influenced by Hevesy, undertook analogous experiments on lead[5–8], bismuth[9,10] and polonium[11–14]. Mention should also be made of the work on the distribution of radium in animals, which has been going on almost since the beginning of the century, but which is less biochemical than medical or toxicological[14–17] (*cf.* p. 145). Finally, some interesting investigations on the metabolism of the inert gas radon were published quite early[18–21].

A fast expansion of tracer techniques began only in the mid-thirties. This was caused principally by the discovery of the natural occurrence of the stable heavy hydrogen by Urey, the development of procedures for enriching stable isotopes, the construction of machines for transmutation by Cockcroft and Lawrence, and the discovery of artificial radioactivity by Joliot and I. Curie. These developments led almost immediately to an extension of isotopic methods from elements which have relatively little interest biologically, like lead, bismuth, thorium and radium, to the lighter elements which play predominant roles in the normal economy of living things.

Hevesy was the first to realize the importance of deuterium and of artificial radioisotopes for biochemistry. Two papers on heavy hydrogen appeared as early as 1934[22, 23]; the heavy hydrogen was provided by Urey. (Pioneering work with heavy hydrogen was carried out in biochemical fields from 1935 onwards particularly by R. Schoenheimer, and brilliant successes were achieved[24]). There followed in 1935 a paper on 'Radioactive Tracers in the Study of Phosphorus Metabolism in the Rat'[25]. Nevertheless, a list of methods useful in the elucidation of reaction mechanisms compiled by a prominent biochemist as late as 1939 still failed to mention the isotopic method[26]. A general increase in the interest in isotopic methods came only after the second World War. This is attested briefly by Table 1 (below) which refers to the leading biochemical journals.

TABLE 1

FRACTION OF TOTAL NUMBER OF STUDIES PERFORMED WHICH USED LABELED ATOMS

Journal	*Volume and year*	*Radioactive isotopes* (%)	*Stable isotopes* (%)
J. Biol. Chem.	157 (1945)	1	4
(U.S.)	223 (1956)	39	7
Biochem. J.	39 (1945)	0	0
(Gt. Britain)	63 (1956)	18	1
Biochimia	11 (1946)	0	0
(Russia)	21 (1956)	7	1
Biochem. Z.	318 (1947/48)	0	0
(Germany)	328 (1956/57)	7	0

The most important point about isotopic methods is the possibility of distinction between atoms of the same element, that is, between the labeled and the unlabeled atoms. Hence it is possible to trace the movement (in the broadest sense) of the atoms in a system which already has a stationary concentration of atoms of the same chemical sort. Such an investigation is possible with no other method. Systems with stationary concentrations are obviously highly significant in biochemistry, and the investigation of metabolism in just such systems constitutes the central problem of biochemistry.

Stable isotopes are, in principle, capable of yielding the same results as radioactive isotopes. It is certainly technically more difficult to use them, but nevertheless it is necessary for those elements which have no suitable radioisotopes, especially nitrogen and oxygen[27]. Hydrogen and carbon can be labeled with stable or with radioactive atoms. No matter which isotope is used, the same results are obtained (disregarding the isotope effect; Chapter V). Even in the case of the latter two elements, however, the use of the radioactive labels is becoming increasingly frequent.

Stable isotopes of elements, which have useful radioactive isotopes, remain indispensable, or nearly so, only in three limited fields of application. (*1*) Because the concentrations of stable isotopes can be measured—with the mass spectrometer—more accurately than those of radioactive isotopes, they are often used for the determination of the isotope effect (Chapter V). (*2*) Because they can be employed in higher concentrations than radioactive isotopes, they permit the determination of reaction mechanisms by procedures in which all or nearly all of the molecules must be labeled (p. 42)[28]. (*3*) In certain experiments double labeling is necessary; in some such cases only a stable isotope can be used as the second label (p. 40).

Because of the qualifications of the author, the present book is devoted to radioactively labeled atoms, and does not discuss the techniques employed with stable isotopes. In the following, *radioactive* isotopes are meant when 'isotopic methods' are mentioned. Let us re-emphasize, however, that the majority of the results can in principle be obtained just as well with stable as with radioactive isotopes, so that a sharp distinction would be unnatural. Indeed, a not inconsiderable part of the investigations described in Chapters XI–XVI—especially the earlier ones—were carried out with stable isotopes.

2. Advantages and Limitations of Radioisotope Methods

There are several reasons for the preference given to radioactive isotopes. We note first of all the extreme sensitivity with which radioactive elements can be detected, permitting the observation of small numbers of atoms; hence work with radioelements can be carried out cheaply. Further, since the measurement of radioactivity is carried out by means of the external effects of the rays, the measurement is often performed on the intact system (non-destructive investigation). Finally, the determinations require relatively little time and effort, once the proper equipment has been obtained.

In comparison to these tremendous advantages of the radioisotope method, its single serious disadvantage, its low accuracy, is of slight importance. Measurements of the intensity of radiation in biochemical practice rarely have an accuracy greater than 1–2%, since the sensitivity of the apparatus is subject to certain fluctuations and it is difficult to reproduce the positioning of the sample precisely. Radiochemical methods are therefore surpassed in accuracy by other chemical techniques. However, it is exactly in the biochemical field that—aside from the abovementioned determination of the isotope effect—the requirement for extreme accuracy of individual determinations seldom arises. The biological variability makes any great precision seem pointless.

The application of the radioisotopic methods to biochemistry can also be called 'radiobiochemistry'. This term is derived from the concept of 'radiochemistry'. According to the fortunate definition of Paneth, radiochemistry is the chemistry of those substances which are detected by their (radioactive) radiations. The fundamentals of radiochemistry will be discussed in Chapter III.

Contrary to radiochemistry (Radiochemie, radiochimie, radiochimiya), radiation chemistry (Strahlenchemie, chimie des radiations, radiatsionnaya chimiya) is the science of the chemical effects of radioactive rays. Logically, then, one designates as radiation biology the important branch of investigation which deals systematically with the changes in living matter brought about by radiation, especially that from radioactive materials. The recommended nomenclature is then:

	In chemistry	*In biology*
Investigations with labeled atoms	radiochemistry	
Investigations of radiation effects	radiation chemistry	radiation biology

If a particular term for biological investigations with radioactive tracers is desired, the word radiobiology, analogous to radiochemistry and radiobiochemistry, suggests itself. Unfortunately, however, this term is sometimes employed as a synonym for radiation biology.

Within the compass of this book, therefore, radioactive atoms will be used as labels only, and the radiation emitted by them will be used for their analytical detection only. Hence, in this book it will always be tacitly assumed that the rays produce no disturbing effects in the materials investigated. Occasionally biochemists about to undertake work with radioisotopes entertain fear that this assumption may be unjustified. These fears are groundless when the work is properly carried out. Although one should remain aware of the theoretical possibility of such disturbances, one can in practice keep the amount of the radioelement, and hence the intensity of its radiation, below the level at which detectable biological effects appear. The beginner in radiobiochemistry is herewith assured emphatically that a clean, sharp separation between this field and that of radiation biology is not only desirable, but easily possible. Some fundamental principles of radiation chemistry and radiation biology will be given in Chapters VI and VII.

Another problem of the isotope method lies in the possibility of isotope effects. It will be shown in Chapter V, however, that isotope effects can seriously disturb biochemical work only in the case of hydrogen. Even with this element there are ways and means of avoiding errors due to isotope effects (p. 40). In conclusion, therefore, it may be asserted that in practice the useful scope of isotopic methods is limited neither by radiation effects nor by isotope effects.

3. Plan of the Book

In view of the tremendous extent of contemporary biochemical work and the great portion of it which involves isotopic methods, it cannot be the purpose of our book to catalogue the most important results obtained by these methods. Moreover, a logical separation of the results of isotopic methods from those obtained by other methods in the same field of biochemistry would be impossible—witness, for example, the problems of photosynthesis.

For these reasons the author considers it his task to introduce the reader to the methodology of biochemical work with labeled atoms. After the principles of work with radioactive substances and of radiochemistry have been presented, the distinctive characteristics of the method will be discussed on the basis of suitable examples. The examples will be taken from the principal fields of biochemistry, and it will be left to the reader, if he specializes in a different sphere, to read for himself the literature concerning his own subject, or to decide how to adapt the procedures to his own field. Completeness in any direction will not be attempted.

A certain difficulty attends the attempt to draw a line between biochemistry and physiology. In this connection, we are guided by the words of Gowland Hopkins... 'What we have come to call General Physiology is now a rapidly advancing branch of experimental enquiry, and it is perhaps less easy to justify an attempt to distinguish between its activities and those of modern biochemistry than between the latter and those of classical physiology. There must be... an overlap in their fields which is entirely desirable. Yet there is still a distinction which seems to be real. Physiology as ordinarily understood is chiefly concerned in every case with the visible functioning of the organs; biochemistry rather with the molecular events which are associated with these visible activities'[29]. In this book, too, some consideration of physiology is inevitable, but specifically medical questions will be avoided as much as possible.

A knowledge of the basic facts of radioactivity is taken for granted. It is presumed, therefore, that the reader is familiar with the concepts of radioactive disintegration, of α-, β-, and γ-rays, and of half-life. A number of elementary books are available to refresh the memory and broaden the knowledge of such matters[30–41].

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CHAPTER II

RADIOELEMENTS IN BIOCHEMISTRY

1. Natural and Artificial Radioisotopes

Because of their fundamental importance, and because of the confusion which exists with respect to them, it appears necessary at this point to define the concepts of 'activity' and 'intensity'. The (absolute) activity is the number of disintegrations in the sample per unit time. The activity has the dimension sec^{-1}. The activity thus defined is independent of the type or the energy of the emissions. For example, samples of radioactive hydrogen and radioactive phosphorus have the same activity if equal numbers of disintegrations take place in each in the same period of time, despite the fact that the average energy of each disintegration is one hundred times smaller in the first sample than in the second. The derived concepts of specific activity and relative activity will be introduced later.

The need for a general unit of activity has arisen. The activities of commercial radioelements, for example, must be expressed in definite absolute units. An activity of one curie (C) was originally ascribed to a source which underwent as many disintegrations per unit time as 1 g of radium (free of decay products)—approximately 3.7×10^{10} per second. This definition suffered from the fact that the activity of radium is not known very exactly. The curie has therefore recently been assigned a round value: one curie is exactly 3.7×10^{10} disintegrations per second. The activity of a gram of radium is therefore no longer exactly 1 C. One one-thousandth of a curie is a millicurie (mC), and one one-millionth is one microcurie (μC).

The number of rays striking unit surface per unit time is termed the intensity. The intensity is by this definition also independent of the type and energy of the individual emissions; its dimensions are $cm^{-2}\ sec^{-1}$. In practice the activity of a sample is usually measured by the intensity of its rays. This intensity is determined with the aid of a particular instrument located at a definite point.

Radioactive isotopes of all the chemical elements are known without exception. Several elements (Nos. 43, 61 and 84–101) exist only in the form of radioactive isotopes. In some important cases, *e.g.* oxygen and nitrogen, all the radioisotopes are so short-lived that they are only of little use; the biochemist is fortunate, however, that the elements most important to him, hydrogen and carbon, possess isotopes with sufficiently long half-lives. A table of the radioisotopes most important for biochemical studies appears at the end of this book (Table 13, p. 324).

In some cases the naturally occuring radioelements are still employed today. For experiments with lead, bismuth, polonium, radium, francium, radon, actinium, thorium and uranium, for example, the natural isotopes are often employed. It is easy and cheap, for instance, to label lead for metabolic experiments either with long-lived radium D (lead 210; half-life $\tau = 19.4$ years) or with short-lived thorium B (lead 212; $\tau = 10.6$ h). The former is readily available in spent radon capsules from cancer hospitals, and the latter can be isolated easily and cleanly from thorium salts[1]. A general outline of chemical methods of separating the desired radioelements from accompanying materials will be given in Chapter III.

Much more important at the present time are the artificial radioisotopes. These are usually obtained by a reaction of slow neutrons with atoms. The atomic nuclei absorb slow neutrons better ('with a larger cross-section' as the physicists say) than fast neutrons. In most cases—but not in all—the slow neutrons are simply added to the atomic nuclei. The reaction is then designated as 'radiative neutron capture', or as a (n, γ)-reaction; a neutron enters the nucleus, and the binding energy is released as a γ-ray, or a cascade of γ-rays, without a particle being emitted. β-active isotopes of the original element are often produced by such neutron capture. For example:

$$^{23}_{11}\mathrm{Na} + ^{1}_{0}\mathrm{n} = ^{24}_{11}\mathrm{Na} + \gamma$$

or, in abbreviated form:

$$^{23}_{11}\mathrm{Na}\ (\mathrm{n}, \gamma)\ ^{24}_{11}\mathrm{Na}\ \text{(instantaneous reaction)}$$

followed by:

$$^{24}_{11}\mathrm{Na} \xrightarrow{\beta} ^{24}_{12}\mathrm{Mg}\ \text{(stable)};\ \tau = 15.0\ \mathrm{h}$$

A short-lived radioisotope is $^{128}\mathrm{I}$ which is formed by bombardment of ordinary iodine with slow neutrons:

$$^{127}_{53}\mathrm{I}\ (\mathrm{n}, \gamma)\ ^{128}_{53}\mathrm{I};\ ^{128}_{53}\mathrm{I} \xrightarrow{\beta} ^{128}_{54}\mathrm{Xe}\ \text{(stable)}$$

The half-life of this radioisotope is only 25 min, so that after 4 hours 99.9% of the radioiodine has disappeared. Very short-lived isotopes must be prepared at the place where they are to be used[2], and, of course, cannot be used at all for experiments of long duration.

All neutron sources produce fast neutrons. These are slowed down by so-called moderators, *i.e.*, the neutrons are allowed to collide with the nuclei of light atoms, and thereby transfer a part of their energy to them. Slow neutrons finally result. The moderators used in the laboratory are chiefly water and paraffin wax; the hydrogen in these substances is the effective moderator. Neutrons which have been slowed down until they have not greater kinetic energy than the molecules of the environment, are termed 'thermal' neutrons. Their kinetic energy corresponds only to that of thermal motion.

In the "natural sources", α- or γ-rays from radioactive elements are used to release neutrons from a nucleus whose 'last' neutron is only loosely bound. The most suitable of these elements is beryllium, which consists entirely of the isotope of mass 9. The so-called α-neutron sources use the reaction ${}^{9}_{4}\mathrm{Be}\ (\alpha, \mathrm{n})\ {}^{12}_{6}\mathrm{C}$. The α-particles are furnished by radium (and its decay products), by isolated radon (and its decay products) or by polonium, the radioelement being mixed as intimately as possible with powdered beryllium. Radium must of course always be used in the form of a salt. The sources are contained in sealed metal or glass capsules.

The γ-neutron sources are based upon the reaction ${}^{9}_{4}\mathrm{Be}\ (\gamma, \mathrm{n})\ 2{}^{4}_{2}\mathrm{He}$. They must contain a radioactive element which emits γ-rays with an energy of at least 1.63 MeV, the binding energy of the last neutron in beryllium. Here again radium or radon, together with their decay products, can be used, but the artificial nuclide ${}^{124}\mathrm{Sb}$ is also used. Although the number of neutrons obtainable from natural sources is always small, they do have certain advantages, such as constancy of neutron emission, cheapness, reliability, relative safety, and convenience.

A large number of neutrons are produced by accelerators, *i.e.*, devices in which nuclear transformations are effected by use of electrical energy. The chief types of such accelerators are the Cockcroft-Walton and Van de Graaf machines and the cyclotron. All of these can serve as neutron sources, if the ions set in motion by the powerful electric fields are allowed to hit suitable targets from which they liberate neutrons by nuclear reactions. The reactions most frequently used are

$${}^{2}_{1}\mathrm{D} + {}^{2}_{1}\mathrm{D} = {}^{3}_{2}\mathrm{He} + {}^{1}_{0}\mathrm{n};\ \text{(or, briefly)}\ {}^{2}_{1}\mathrm{D}\ (\mathrm{d, n})\ {}^{3}_{2}\mathrm{He}$$

and also

$${}^{7}_{3}\mathrm{Li}\ (\mathrm{d, n})\ 2{}^{4}_{2}\mathrm{He}$$

and

$${}^{9}_{4}\mathrm{Be}\ (\mathrm{d, n})\ {}^{10}_{5}\mathrm{B}$$

with deuterons as the projectiles; in certain cases bombardment with protons can also be employed.

Usually, however, slow neutrons from a uranium reactor (pile) are used.

Many of these reactors have access ports through which samples can be introduced into the interior of the reactor and exposed to strong neutron fluxes. Atomic energy laboratories, such as those at Oak Ridge, Tennessee, at Harwell in England, or at Kjeller in Norway offer radioisotopes produced in this way at low prices. In Table 13 (see p. 324) the radioisotopes which are most suitably obtained from the pile are marked with the letter R.

The activity of the radioelements produced is proportional to the neutron flux employed, that is, to the number of effective neutrons impinging on unit surface per unit time. Since in most cases it is the slow neutrons which react, it is their flux which is usually decisive. The flux of slow neutrons from an electric neutron generator is in practice much smaller than that in a uranium pile (Table 2).

TABLE 2

FLUX OF SLOW NEUTRONS (TYPICAL VALUES)

	Flux (cm^{-2} sec^{-1})
Material testing reactor	5×10^{14}
BEPO reactor at Harwell (maximum)	10^{12}
BEPO (average)	10^{11}
Accelerator	10^{6} to 10^{9}
Natural sources	$< 10^{5}$

The useful nuclides, long-lived carbon (^{14}C), tritium (^{3}H or T) and ^{35}S, are prepared by nuclear reactions other than neutron capture. When slow neutrons are absorbed by nitrogen, γ-emission does not take place, but instead proton emission occurs, the reaction being $^{14}_{7}N$ (n, p) $^{14}_{6}C$. In practice, a compound of nitrogen, such as an ammonium salt, a nitrate, or the compact beryllium nitride, is exposed in the pile. Most of the radiocarbon formed combines with oxygen at the moment of formation; it is then present as radioactive carbon dioxide, which is pumped off, combined with barium hydroxide, and sold as barium carbonate. Radiocarbon present in other chemical forms is first oxidized.

The important radiohydrogen (tritium; $\tau = 12.3$ years) is usually also produced with slow neutrons. The nuclear reaction is:

$$^{6}_{3}\mathrm{Li}\ (\mathrm{n}, \alpha)\ ^{3}_{1}\mathrm{T}$$

^{35}S ($\tau = 87$ days) is formed by the reaction:

$$^{35}_{17}\mathrm{Cl}\ (\mathrm{n}, \mathrm{p})\ ^{35}_{16}\mathrm{S}$$

Finally, numerous radioelements are produced in the fission of uranium by slow neutrons [(n, f)-reaction]. The fission products occur in enormous quantities in exposed reactor fuel. All elements with atomic numbers 30 to 65 (gallium to terbium) have been found among the fission products. For example, ^{131}I, much used in biochemistry, is a fission product.

To obtain the very important nuclide ^{32}P ($\tau = 14.2$ days), the reaction of sulfur with fast neutrons

$$^{32}_{16}S\ (n, p)\ ^{32}_{15}P$$

is employed. The yield is quite good, and the ^{32}P is obtained free of inactive phosphorus. Free sulfur is irradiated in the reactor, the reaction mass is then fused and extracted with water or with acid[3].

The isotope ^{33}P ($\tau = 25$ days, *cf.*[4,5] for methods of preparation) has recently become important for purposes of double-labeling (p. 39). ^{33}P can be distinguished from ^{32}P since the energy of its β-rays is much smaller. For the same reason, ^{33}P is particularly well suited to autoradiography[6] (p. 87).

Some nuclides important for biochemical work cannot be obtained by the action of neutrons on atomic nuclei. These materials are prepared with the aid of accelerators: suitable target substances are transformed into the required nuclides by bombardment with fast ions. The yields in these reactions are always low, since the ions (in contrast to neutrons) are repelled by the positively charged atomic nuclei and hence are able to react only if they have not lost much energy during their passage through the target material. The repulsion is particularly pronounced when fairly heavy or very heavy, *i.e.*, highly charged, nuclei are bombarded. In any case, the nuclear reaction occurs only in a thin layer at the surface of the target.

An example is the long-lived ^{54}Mn ($\tau = 290$ days) which is necessary for biological experiments with manganese extending over long periods. This nuclide is obtained by deuteron bombardment of natural chromium, which apparently causes the reaction $^{53}_{24}Cr\ (d, n)\ ^{54}_{25}Mn$ to occur. Another nuclide important in biochemistry, which can be produced only with the aid of an accelerator, is the long-lived ^{22}Na ($\tau = 2.6$ years). The reaction is $^{24}_{12}Mg\ (d, \alpha)\ ^{22}_{11}Na$.

2. Specific Activity

The method of preparing radioelements by the simple capture of slow neutrons [(n, γ)-reaction] is the most commonly used one, and is convenient, but has one great disadvantage. The nuclear species which captures the neutron and the radioactive species are isotopes and cannot be separated chemically. The inactive element must be carried along when the radioactive nuclide is used; it is therefore referred to as a 'carrier'. The specific activity (activity per unit mass) is therefore always relatively small in the case of radioelements prepared by slow-neutron capture. In biological work this is an unfavorable circumstance, since the introduction of large amounts of material can disturb the natural metabolism.

Radioactive nuclei which are produced by slow neutron reactions other than capture, especially ^{14}C, ^{3}H, and ^{35}S, are fortunate exceptions. The long-lived radiocarbon, for example, can be separated by chemical means from the parent nitrogen compounds. Ideally, pure 100% radiocarbon—*i.e.*, carbon of maximal specific activity—could be obtained.

In practice, however, the radiocarbon is always contaminated with inactive carbon (^{12}C and ^{13}C), and its specific activity is usually approximately ten times less than the maximum possible value. One may say that the 'isotopic purity' is 10%. Nonetheless, its specific activity is very much greater than that of ^{14}C obtained by the reaction $^{13}_{6}\mathrm{C}\,(\mathrm{n}, \gamma)\,^{14}_{6}\mathrm{C}$, in which the radiocarbon is formed by neutron capture. The specific activity of radioelements prepared with fast neutrons is also high, since they are not produced by the (n, γ)-reaction.

Similarly, radioelements formed by bombardment of a target with charged particles are free of carrier, or at least poor in carrier. When a charged particle is taken up, the nuclear charge, and hence the chemical nature of the atom, are altered. As a result, a chemical separation of the radioelement from its parent substance becomes possible. For example, the long-lived sodium mentioned above is easily separated from the magnesium by converting the magnesium to the hydroxide, and extracting this sparingly-soluble substance with water. The carrier—stable sodium—then consists only of the very small quantity originally present in the magnesium as an impurity; the specific activity is therefore very high.

The radioelements which can be prepared from non-isotopic starting materials, and consequently can be obtained carrier-free or essentially so, are marked with an asterisk in Table 13.

Finally, there exists a special procedure by means of which in some cases even the radionuclides obtained from their stable isotopes by neutron capture can be prepared in a carrier-poor state. This procedure takes advantage of the so-called Szilard(-Chalmers) effect—a phenomenon in the field of 'hot atom chemistry'. Elements to which this procedure is applicable are not specially indicated in Table 13; the reader is instead referred to the reviews in the literature[3, 7–11].

The Szilard effect depends basically on the fact that a portion of the binding energy of the neutron serves to release the atom concerned from the molecule, that is, to convert it to a different chemical form. The difference between the chemical states of the same element in the parent substance (stable) and the product (radioactive) can be utilized for the separation.

In their classical work (1934) the discoverers of the effect irradiated ethyl iodide and observed that the radioactive ^{128}I obtained by neutron capture from ^{127}I was present chiefly as free iodine or as iodide ion in chemical equilibrium with free iodine. This free iodine could be separated from the main mass of organic (covalently-bound) iodine by extraction with water or alkali. Besides the halogens, most of the heavy metals and also some other elements are suitable for application of the Szilard effect.

3. Radioactive Purity

The user of radioelements is naturally concerned whether or not they contain admixtures of different substances which are also radioactive—in other words, with their radioactive purity. Even extremely small quantities of strongly radioactive impurities can cause disturbances. When the impurity consists of the 'wrong' isotope of the 'right' element, it can be detected by changes in the decay curve and in the properties of the radiation. An important case is that of the two iron isotopes 55 and 59 ($\tau = 2.9$ years and 45 days, respectively). Since a subsequent separation is impossible, it is necessary either to prevent the wrong isotope from being introduced in the first place (or at least, to keep it down to a harmless level) or to take into account the presence of the second isotope during the measurements, and in the interpretation of the measurements.

If an active isotope of a foreign element is involved, it will behave (in metabolic experiments for example) differently from the element being studied. For this reason, such experiments impose very strict requirements with respect to freedom from extraneous radioactive elements—far stricter, indeed, than the usual requirements for the chemical purity of reagents[12].

When a radioelement proves to be contaminated in this sense, it must be purified. The preparation is subjected to chemical reactions in which the desired element and the impurity behave differently; selective precipitation or chromatography (see Chapter III) may be employed, for example. Before precipitation appreciable quantities of the inactive impurity are often added as carrier (see p. 18) in order to suppress adsorption which may carry the radioactive impurity along with the element being purified; the adsorbed material will then consist almost entirely of the relatively harmless inactive isotopes. Analogous steps must be taken in other separation procedures.

The relatively noble element polonium, for example, can be selectively deposited at the cathode at low voltages. Radiolead (radium D) which is admixed with the polonium, as obtained from uranium ore or from spent radon capsules, is easily adsorbed on the electrolytic deposit in trace amounts. This adsorption is suppressed by the addition of inactive lead salts, very small quantities being sufficient for this purpose. The presence of minimal quantities of this inactive lead in the polonium preparation is probably not harmful.

There are however cases where a radioelement continually contaminates itself. It must then be re-purified shortly before use, or such re-purification may even be impossible. Such self-contamination occurs when the radioactive element decays to give another radioactive element. The daughter element continuously comes into radioactive equilibrium with the parent, *i.e.*, after separation the daughter element is formed again, until the rates of formation and decay are in equilibrium. This occurs when the activities of parent and daughter are approximately equal. The radioactive equilibrium is determined by the half-life of the daughter. Radioactive chains occur in the three natural radioactive series, which start with uranium, actino-uranium and thorium. Many of the fission products also give rise to radioactive chains.

However, even where radioactive decay products exist, definite conclusions can often be drawn from the experiments. For example, pure thorium B ($\tau = 10.6$ h), the only lead isotope of the thorium series, gives rise to the bismuth isotope, thorium C, which is also a β-emitter ($\tau = 1$ h). It is well therefore to wait before counting the samples (organs of animals which have been fed with thorium B, or lead salts isolated from them) until the equilibrium between thorium B and thorium C has re-established itself, *i.e.*, for several hours. Not only the activity of the thorium B, but also that of the thorium C is then determined by the amount of lead isotope present in the preparation at the end of the experiment. Similarly, the important fission product of uranium, ^{140}Ba, forms ^{140}La by β-emission. The half-lives are in this case 300 h and 44 h. Barium and lanthanum thus come to equilibrium after a period of several days.

Finally, the purity of a radioactive preparation may be insufficient, even if it contains only the 'right' isotope, if this isotope is present partly in the form of the 'wrong' chemical compound, and no exchange occurs between the two forms[13]. It has been observed, for instance, that phosphate is reduced when neutrons are captured by the phosphorus[14–17], and radioactive phosphite and other oxygen-deficient radioactive compounds are obtained. Some elements have a strong tendency to change into the colloidal form ('radiocolloids', see p. 24) and then not to exchange with the simple ions[3, 18]. For example, when dissolved salts of zirconium are to be labeled by the addition of traces of radiozirconium, it must be verified that the radioelement has not changed into the colloidal state.

References p. 16.

During radiosyntheses, a host of labeled substances can arise simultaneously (Chapter IV). When radioactive compounds are used for tracer work in organic chemistry or in the study of metabolism, very grave errors can arise if care is not taken to have all of the labeled material in the same chemical form. The freedom of the labeled material from radioactive contaminants is established by submitting it to various purification procedures, and then re-determining its specific activity. If the material is pure, further purification cannot alter its specific activity. A very effective procedure, in addition to distillation, chromatography and precipitation, is (especially for organic materials) counter-current distribution[19]. The possibility of contamination by spontaneous decomposition is discussed in Chapter VI.

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CHAPTER III

PRINCIPLES OF RADIOCHEMISTRY

1. The Exchange of Atoms

Only with the aid of isotopes can it be determined whether atoms are exchanged between two different compounds. In this way it was established as early as 1920 that lead chloride in solutions exchanges lead atoms with lead nitrate and even with salts of tetravalent lead, but not with lead tetraphenyl[1]. We can say in general that elements which form electrovalent bonds are capable of exchange, while no exchange can be observed between similar atoms in two molecules, or even in the same molecule, if one or both are held by firm covalent or complex bonds. Whether the bond in any given case is a firm one can often be predicted on theoretical grounds, but is decided with isotopes. Some compounds exchange their atoms at elevated temperatures even though the exchange is immeasurably slow at room temperature. The exchange can also be accelerated by catalysts.

The 'active' hydrogen of alcohols, acids, and amines, for example, is capable of exchange under the usual experimental conditions. So is the hydrogen of methylene if it can undergo enolization. Oxygen exchanges between the two positions of the carboxyl group. Carbon atoms as such are never exchanged, not even within a chain, but carbon dioxide can exchange as a whole between the gas phase and a carboxyl group when suitable catalysts (enzymes) are present. The possibility of chemical exchange must be carefully considered in the preparation of labeled compounds and in metabolic investigations. Reviews of exchange reactions may be found in the literature[2–7]. It is also possible to measure the exchange of atoms, ions, or entire molecules between different phases as they cross phase boundaries without changing their chemical nature (Chapter X).

The investigation of exchange phenomena has found an interesting application in the study of the spatial structure of proteins. Whereas simple peptides dissolved in heavy water quickly exchange all of the hydrogen bound to nitrogen or oxygen (but none of that bound to carbon) with deuterium, insulin exchanges only a part of the 'active' hydrogen with some rapidity. It is concluded that part of the hydrogen, which is in principle exchangeable, is sterically hindered[8, 9]. Similar investigations have been made with other proteins[10, 12] and peptides[11] as well as with nucleoproteins[12]. Radioisotopes have not hitherto been used, however.

The non-exchange of covalently-held atoms, particularly carbon atoms, is utilised in the investigation of reaction mechanisms[13–16]. Two examples from the field of organic chemistry may be cited. (*a*) On thermal decomposition of the ester of an α-ketoacid the carbon monoxide

$$RCOCOOC_2H_5 \rightarrow RCOOC_2H_5 + CO$$

produced is labeled only when the ester was labeled in the carboxyl group. Apparently the carbon monoxide arises only from this group[17]. (*b*) When ethylamine labeled in the methylene group is treated with nitrous acid, a (small) part of the radiocarbon migrates to the methyl

$$CH_3CH_2NH_2 + HNO_2 \rightarrow CH_3CH_2OH + N_2 + H_2O$$

group. Thus the mechanism of this seemingly so simple reaction must really be complicated[18, 19]. Many examples involving the mechanisms of biochemical reactions will be found in the specialized part of this book.

The relation between exchange phenomena and the 'dynamic state of the body constituents' will be dealt with in Chapter XI.

2. The Use of Carriers

In radiobiochemistry, labeled substances must be separated from one another and from unlabeled substances. In these separations the chemical behavior of the radioactive isotopes is fundamentally like that of the inactive isotopes of the same elements. In practice, however, certain peculiarities arise in chemical work with radioelements. The rules which here prevail constitute the chief subject of radiochemistry (p. 4). An outline of radiochemistry may be found in the literature[3, 5, 7, 20, 21, 22, 23, 24].

A most important characteristic of radiochemistry is that the radioelements are often present only in imponderable quantities—so-called traces—even though they possess great activity. The traces naturally behave differently from weighable quantities. In order to avoid such differences, which can occasion unwelcome surprises, it is possible in some cases to add weighable quantities of 'carriers'. The radioelements can then always be handled by the usual chemical procedures.

By 'carriers' the radiochemist means all inactive elements or compounds which are present in weighable quantities and whose chemical properties are identical with, or at least very similar to, those of the radioelement or its compounds. Complete identity of course occurs only when the element or its compounds are themselves used as carriers. The use of the inactive isotope of the same element entails the inevitable disadvantage that the carrier can under no circumstances be subsequently removed and the specific activity of the radioelement is irrevocably reduced (isotopic dilution). If one wishes to avoid this, it is best to use as carrier a similar, but non-identical element; after it has served its purpose, it can be removed by suitable reactions. Similar considerations apply to the selection of compounds to be used as carriers of labeled compounds.

For example, in the precipitation of imponderable traces of radiolead hydroxide from acid solutions with ammonia, lead or aluminum ions can be added as carriers in order to obtain a weighable and visible precipitate. This precipitate then consists of course mainly of inactive lead hydroxide, or of aluminum hydroxide. The radiolead can later be separated from the aluminum by the familiar method of deposition at the anode as lead dioxide, for example; on the other hand, a chemical separation from inactive carrier lead would be quite impossible.

In work with traces, particular attention must be paid to the possibility of adsorption phenomena, because the quantities of material are so small in relation to the surfaces on which adsorption may occur. Catastrophic losses of carrier-free or carrier-poor radioelements by adsorption onto the walls of vessels, onto filter paper, or onto precipitates have been observed quite often. Such adsorption can be reduced by preventing the solutions or gases from coming into contact with large surfaces.

Better protection is afforded by using weighable quantities of substances which displace the radioelement, that is, hinder its adsorption, by occupying the available adsorbing sites. For example, if one is willing to accept isotopic dilution, the adsorption of radionickel ions on the walls of the vessel can be lowered practically to zero by adding weighable quantities of inactive nickel ion; in this case, naturally, most of the nickel adsorbed is inactive rather than active. On the other hand, if the adsorption of the active nickel is to be avoided without diluting the isotope, other metal cations are used to displace the nickel, *e.g.*, cobalt ions. The inactive cobalt can be separated from the active nickel later. Hydrogen ions also displace cations from glass surfaces, so that the loss can be diminished by acidifying the solution.

In biochemical practice, radiochemistry is used either for preparative or for analytical purposes. In the present chapter the preparative point of view is in the foreground, while the analytical applications will be dealt with in Chapter IX. Nevertheless, we would like to emphasize here that the so-called indicator analysis which is to be described later employs essentially the same methods as preparative radiochemistry; quantitative indicator analysis consists in the quantitative preparation of radioactive materials in the pure state, and subsequent measurement of their activity.

3. Partition Procedures and Chromatography

Because of the possibility of adsorption, the radiochemist avoids as far as possible the precipitation procedures which are so much favored by other chemists. He prefers methods in which adsorption plays only a minor role, such as partition between two immiscible liquids, or electrochemical procedures. One method of separation has assumed particular importance, in which adsorption does indeed occur, and in many cases forms the very basis of the procedure, namely, chromatography. Only a few examples of separation procedures can be mentioned here; for the rest, we must refer the reader to the literature on radiochemistry (p.18).

Procedures for separations using pairs of two immiscible solvents have become very important[3, 24, 25, 26]. An example is the much-employed extraction of free iodine from aqueous solution with non-polar solvents after selective oxidation of iodide with nitrous acid. In this procedure chloride and bromide ions are not oxidized and remain in the water together with the cations. Separations by extraction can be made more selective and more effective with the aid of complexing agents.

Electrochemical procedures[24, 25, 27, 28] have also been used in radiobiochemical work. Radioiron, for example, which is employed in the study of the metabolism of blood, can be deposited electrolytically and then measured[29]; see pp. 1 and 14 with regard to polonium. Electrophoretic separations are discussed below.

The possibility of radiochemical separation by selective volatilization can only be mentioned here[3, 24, 25].

Among the chief advantages of chromatography[3, 24, 25, 26, 30–33] in radiobiochemistry are the sharpness of the separations and the applicability of the method to small quantities, even traces, of material. The columns work by virtue of ion exchange, or of secondary valences. As in the case of partition between immiscible solvents, the separation is usually governed by laws which are independent of the absolute quantities of the materials present. Procedures which have been worked out with weighable quantities can therefore be carried over to traces, and vice versa.

The distribution of activity in the column can, in the cases of γ- or hard β-emitters, be determined by direct scanning, without cutting up the column. Usually, however, the different portions of the eluate will be measured. It is also possible to determine the activity of an eluate continuously by allowing it to flow in front of the window or around the wall of a counter. This can be done, for example, by passing the liquid through a spiral tube. In the case of soft β-emitters (^{14}C, ^{35}S) it is advisable to let the eluate flow out in a thin layer and to determine its activity with a flow counter[34] or a scintillation counter[35].

In this way column chromatography permits the preparative or analytical separation into their components of even complicated mixtures of related elements (such as rare earths) or organic molecules (carboxylic acids[36, 37] and phosphatides[38]). Gas chromatography has proved useful in the separation of labeled volatile compounds[39–41], including T- and ^{14}C-labeled compounds[42, 43].

Paper chromatography exhibits similar advantages [32, 44, 45]. For example, extremely small amounts of different metal ions or organic compounds can be separated. Electrolytic desalting[44, 45, 46] is often necessary before chromatography, *e.g.*, when labeled organic compounds are to be isolated from tissue which had been preserved in isotonic saline[47].

So-called radiochromatograms are obtained by passing a Geiger counter with a slit window along the paper strips on which the labeled substances are placed, and thus measuring the relative values of the activity at frequent intervals. The use of windowless counters[34, 48, 49] and of scintillation counters[50] for this purpose has also been described, and devices for the automatic scanning of paper chromatograms have been developed[26, 53–56]. There are also machines for scanning two-dimensional

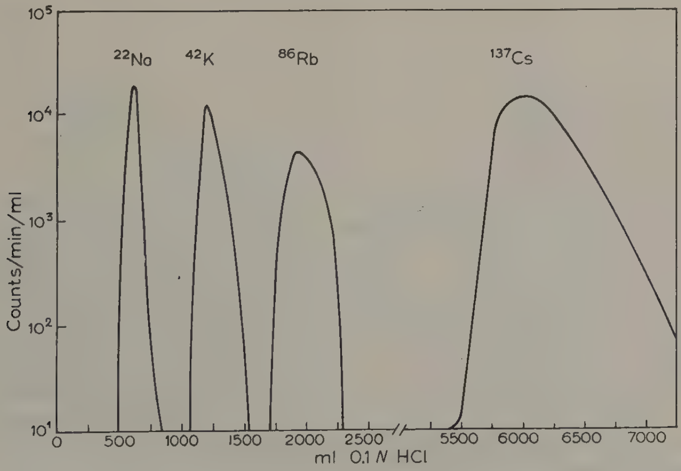


Fig. 1. Separation of alkali ions by elution from cation exchangers[51] (see also [52]). (K and Rb were present in milligram quantities, Na and Cs in unweighable amounts).

chromatograms[57] and for the simultaneous measurement of an entire sheet of paper[58]. The specific activity of the substance can be determined from the size of the active zone and the activity[59].

Fig. 2 shows the radiochromatogram of a mixture of radioglucose, -fructose and -sucrose. This mixture was obtained by radiophotosynthesis (assimilation of radioactive carbon dioxide by a green plant; p. 167). The total quantity of sugars amounted to only about one-tenth of a milligram in this case. Fig. 3 compares the '^{32}P-spectrum' in extracts of normal house flies and of flies poisoned with methyl bromide (p. 119). Other examples of paper chromatography are the separation of hydroxamates of labeled fatty acids[62] and of labeled phosphatides[63, 64].

The radioactive substances on the paper chromatogram can also be determined semi-quantitatively by autoradiography. The paper sheet is pressed against a photographic film, and black spots appear where the radioactive materials are located (see p. 87). This labor-saving procedure

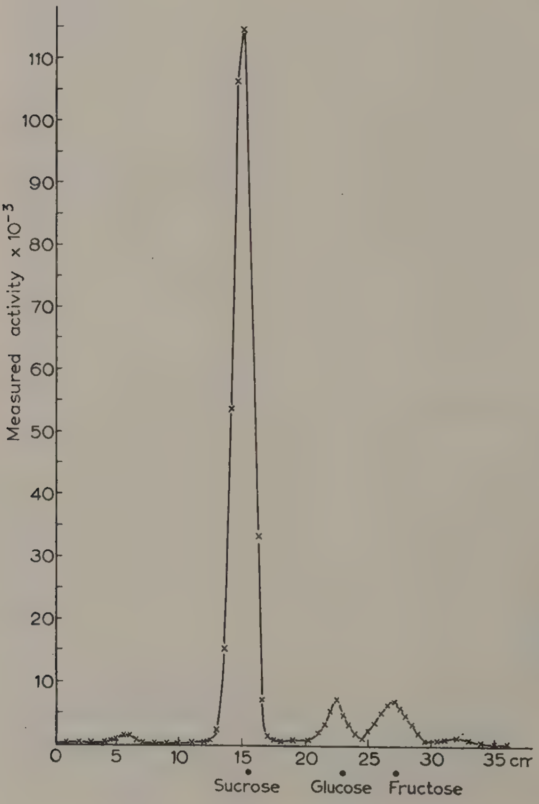


Fig. 2. Radiochromatogram of a mixture of sugars. Scanned with a window counter. The dots represent the locations of inactive sugars which were co-chromatographed and detected by color reactions. Solvent: butanol–acetic acid–water[60].

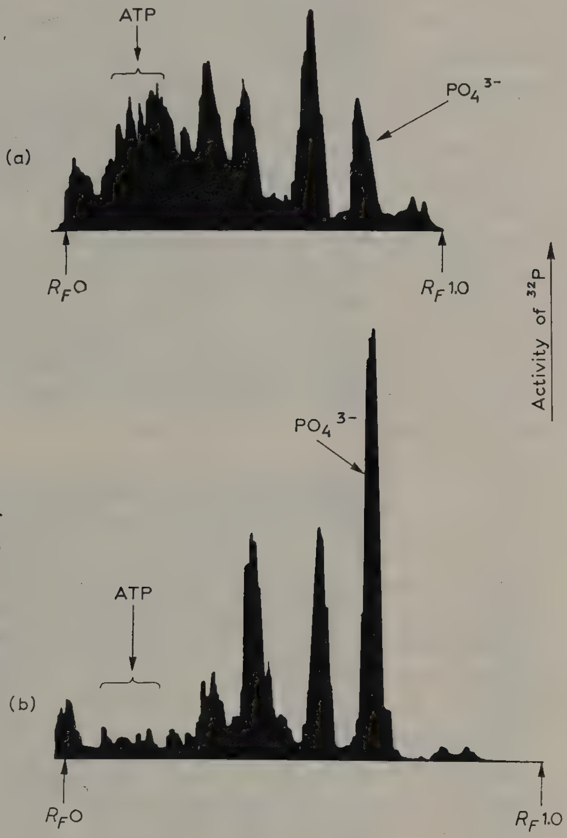


Fig. 3. Radiochromatogram of the acid extract of the muscles of house flies which had been fed labeled phosphate. (a) normal, (b) after poisoning with methyl bromide, an enzyme inhibitor used as an insecticide. Solvent: formic acid–acetone[61].

is especially helpful for the preliminary location of the active materials on a two-dimensional chromatogram. Fig. 19 (p. 169) shows such a'map' of the products of radiophotosynthesis by green algae[65].

Electrophoresis[66] is also much employed in biochemistry[33]. It is carried out in Tiselius cells[67] and also—more often—on carriers. The carrier most frequently used has been paper[44, 68, 68a], but starch[69–71], cellulose[72, 73] and cellulose acetate[74] have their advantages. The pherogram also can be investigated by autoradiography or scanned with a window counter. There are automatic devices for examining pherograms by moving the paper strip past the counter; the same apparatus can be used as with chromatograms,

It is well known that electrophoresis is especially suitable for the separation of soluble proteins. Thus the distribution of radiosulfur[81, 82] among the serum proteins (see p. 231) has been determined by this procedure.

Other examples of applications are the separations of ^{32}P-compounds in blood[75–80], of ^{32}P-nucleic acids [83, 84], and of the mononucleotides obtained by the hydrolysis of labeled nucleic acids (p. 265)[85].

A special electrophoretic method, which can serve for analytical and preparative purposes, is the so-called 'transmigration' electrophoresis (Überwanderungselektrophorese). Several substances which react with one another are placed separately on the strip in such a way that the mixture containing the slower-migrating substance has a lead. During the electrophoresis this substance is overtaken and reaction takes place. The reaction product (which may itself migrate – but usually at a different rate) is detected by its activity (*e.g.* by autoradiography) in its own band, and can be subsequently eluted. This method is suitable for the separation of antibodies from mixtures, using the antigen as the reagent. The technique has also been worked out in two dimensions[86, 87].

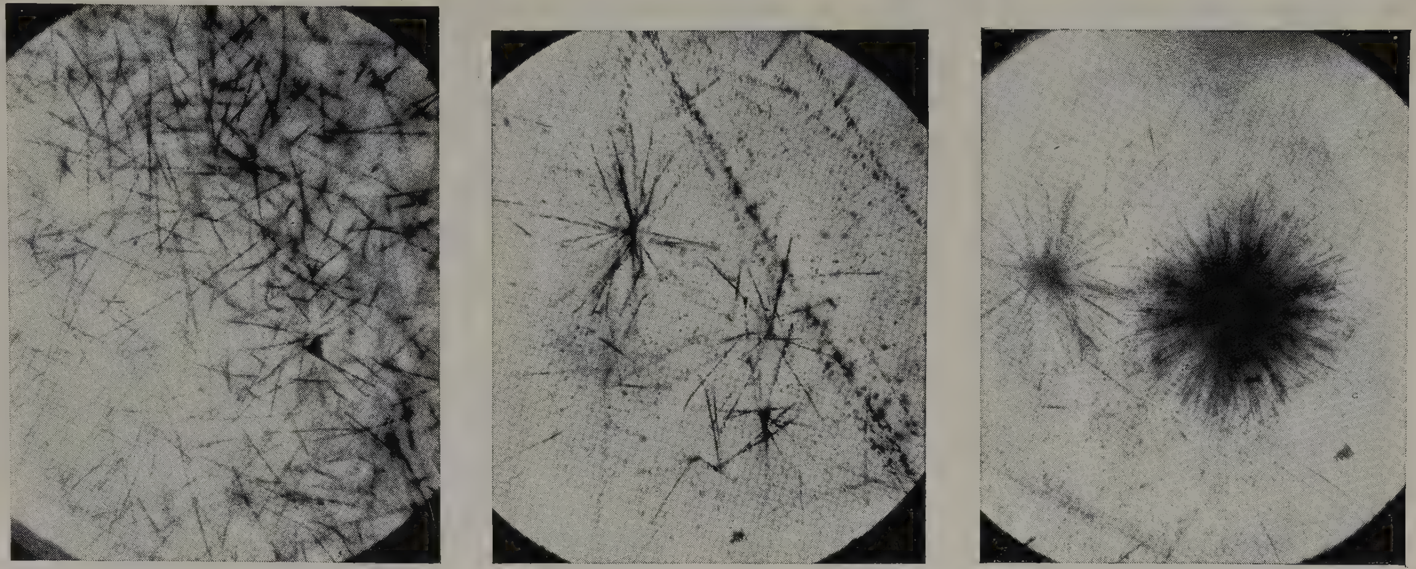
4. Precipitation Methods

Finally, we may discuss the separation procedures which are based on precipitation. In spite of their considerable disadvantages, they remain indispensable[3, 21, 24–26]. Special types of precipitations which have recently become important in radiochemistry are precipitation from homogeneous systems[25, 88] and precipitation on the surface of ion-exchange resins[25].

The radioelements are often present in such high dilution that the solubility product is not reached on the addition of the precipitating agent; hence formation of a precipitate—even an invisible one—is not to be expected. Thus the centrifugation of such a solution would not bring down the active material. Of course, precipitation could be brought about by the addition of isotopic carrier.

Oddly enough, it is found that under some circumstances the precipitation of imponderable and invisible quantities of ions can occur even when, according to the solubility products given in the literature, it should be impossible. For example, radioactive lead hydroxide can be precipitated with ammonia from extremely dilute solutions without the addition of any carrier. When the solution is centrifuged, the active material appears as a precipitate on the walls; on filtration, it does not pass through the filter. Such precipitates are known as 'radiocolloids'; the laws governing their formation are not yet clear[89]. The formation of radiocolloids is often a disturbing factor (p.15). On the other hand, the carrier-free precipitation from extremely dilute solutions can be utilized for purposes of separation. The phenomenon is not limited to radioactive materials, but can only be demonstrated with them (Fig. 4).

Fig. 4. Radiocolloid 'stars' in a nuclear emulsion which had been brought into contact with the 'solution'. (Photographs by F. Epstein and E. Broda, enlarged approx. 500 ×).



a b c

a. Acid solution of polonium. The polonium is present as individual ions.
b. A similar solution after neutralization. The polonium atoms agglomerate to small groups.
c. The neutral solution after heating. The aggregates are much larger.

The precipitation of radioactive ions with non-isotopic carriers has been investigated by Hahn, Fajans and Paneth; to these workers we owe the so-called rules of precipitation, which indicate whether or not traces of radioactive ions will co-precipitate with non-isotopic carriers. These rules are not to be considered as strictly valid laws[24].

Co-precipitation of traces of radioactive ions with weighable amounts of ions, whose charge has the same sign, occurs when the radioactive ion can be built into the crystal lattice of the precipitate. The well-known precipitation of radium with the isomorphous barium salts is an example. Co-precipitation occurs in the absence of isomorphism when a 'tendency' exists toward the formation of an insoluble compound of the radioactive ion with the ion of opposite charge in the precipitate, *i.e.*, if the salt of the radioactive ion and the other ion would precipitate, provided the radioactive element were present in weighable quantities. For example, despite the lack of isomorphism trace amounts of radiolead are easily co-precipitated on silver bromide because lead bromide itself is only sparingly soluble. On the other hand, the radium ion does not co-precipitate since radium bromide is quite soluble.

Analogous results are obtained for the co-precipitation of radioactive anions.

These phenomena are explained by assuming that the co-precipitation is due to a sort of exchange adsorption in which an (incomplete) surface layer of the salt of the radioelement is formed with the ion of opposite charge in the precipitate. The 'tendency toward insolubility' is necessary to the extent that difficultly soluble salts are especially stable so that the radioactive ions remain fixed to those of the precipitate. They are, therefore, only slightly dissociated and hydrated by the water, as would be the case with soluble salts.

However, as has been emphasized by Otto Hahn particularly, there are other influences besides isomorphism and insolubility which play a role in co-precipitation. For example, anions are taken up better by positively-charged than by negatively-charged precipitates; the opposite is true of cations. According to the well-known experiments of Lottermoser, such a positive (or negative) charge arises when the precipitate is formed in the presence of an excess of cations (anions). Silver bromide, for example, precipitates with an excess positive (negative) charge when it is formed from sodium bromide and an excess (deficit) of silver nitrate. Radiolead therefore is better co-precipitated in the latter case.

These rules of precipitation must of course be taken into account not only when co-precipitation is desired, but also when it is to be avoided, as for example in the removal of inactive contaminants from the solution of a radioactive salt by precipitation.

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CHAPTER IV

RADIOSYNTHESIS

1. Procedures Based on Conventional Methods

Radioelements for biochemical applications must most frequently be employed not in the form of free atoms or of ions, but rather as more or less complicated molecules, in which the radioactive atom is covalently bound. The preparation of radioactive compounds may be called ‘radiosynthesis’. In biochemistry, the labeling of carbon is especially important (p. 91), but in many circumstances it is also advantageous to label hydrogen (p. 94) or sulfur (p. 91).

Some of the radiosynthetic methods have no counterparts in the synthesis of inactive compounds, but even those which are derived from traditional synthetic methods exhibit certain peculiarities; these will be the first subject of discussion.

In the *first* place, we must consider ‘radioisomerism’. For example, the chemist has hitherto recognized only one kind of ethyl alcohol, but when radiocarbon is employed, four different ‘radioisomers’ can be distinguished, *viz.*

$$CH_3CH_2OH \qquad \overset{*}{C}H_3CH_2OH \qquad CH_3\overset{*}{C}H_2OH \qquad \overset{*}{C}H_3\overset{*}{C}H_2OH$$

The labeled atom is denoted by asterisks. No account is taken here of the difference between the two stable carbon isotopes 12 and 13.

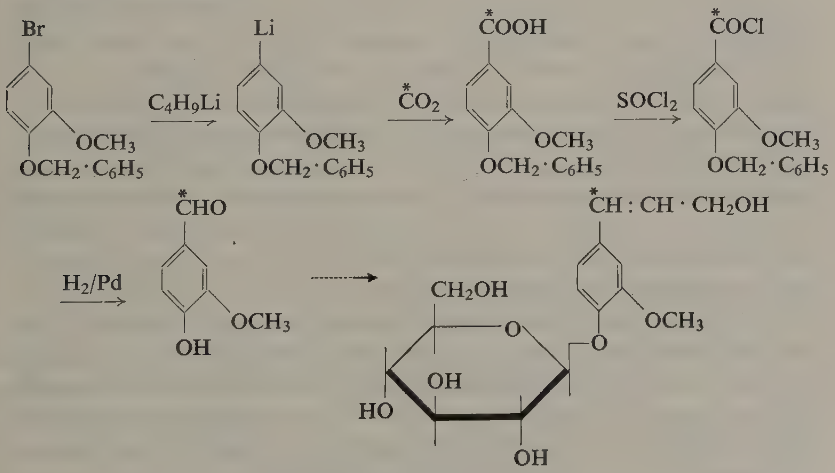
On chemical reaction, such as degradation, or incorporation of the carbon into animal tissues, these various types of alcohol yield fundamentally different products, *i.e.*, products containing the radiocarbon in different locations in the molecule. If the bond between the carbon atoms is broken, one or the other of the products is radioactive. Consequently, if the metabolism is studied on the basis of the radioactivity, quite dissimilar conclusions can be drawn by using different radioisomers. Therefore care must always be taken with regard to the position in the molecule into which the active atom is introduced.

It is easy to see that with increasing complexity of the molecule the number of radioisomers increases rapidly. There are 14 possible radioisomers of benzene and 99 radioisomers of toluene. One may imagine the size of the handbooks of organic chemistry such as 'Beilstein' when radiosynthetic methods have been fully developed.

A particularly interesting form of radioisomerism is radiostereoisomerism. Compounds of the type R_1R_2CDH have proved to be optically active[1], and the same must be true of R_1R_2CTH. Radiostereoisomers naturally behave differently in metabolic experiments.

The position in which a molecule is to be labeled depends upon the use to which it is to be put. In a certain investigation, for example, the fate of one particular methyl group may be of interest, and in others that of a carboxyl group. In no case, however, is it useful to label an atom which is chemically exchanged with an atom present in the biological system, such as the hydrogen of a hydroxyl group.

In the *second* place, radiosyntheses are, in the interests of economy, usually performed with small quantities of active substances. The addition of a large amount of inactive carrier is also impossible if products with high specific activities are desired. Radiosyntheses have been performed with as little as several micromols of material, and syntheses on the millimolar scale are common. It is usually necessary to build special apparatus for work with these small amounts of material; the most important requirement is easy transfer of the materials from one reaction vessel to the next with as little loss as possible. For the synthesis of organic substances it is advantageous to use closed systems in which the reactants can be transferred from place to place by distillation in a high vacuum. Such apparatus has been described in detail, especially for work with radiocarbon[2] and radiohydrogen[3].



Synthesis of radioactive coniferin

As an example of a synthesis which pursues the usual course, and does not employ a closed apparatus, but nevertheless can be carried out with small quantities of material, we mention the synthesis of the radioactive carcinogenic dye butter yellow (dimethylaminoazobenzene). Several milligrams of labeled aniline hydrochloride were diazotized in a simple Emich beaker and coupled with dimethylaniline. The dye was filtered off, dissolved in CCl_4 and chromatographed on alumina (yield 13 mg[4]). Butter yellow labeled in the other benzene ring was also prepared[5] (*cf.* p. 308).

As an example of a synthesis involving many steps and requiring a high vacuum apparatus, we cite the preparation of the glucoside coniferin, a possible intermediate in the formation of lignin in plants[6,7] (p. 303).

In the *third* place, when there is a choice of syntheses, one selects—again in the interest of economy—that which involves the least loss of active material. This is true even when the method has disadvantages in other respects. Accordingly, the radioactive material is often introduced at the latest possible stage, so that the yield is as high as possible with respect to the radioelement. This usually involves the additional advantage that the probability of radioisomerization—*i.e.*, the migration of the radioelement to an undesired position—is less than it would have been if the radioactive material had been introduced at an earlier stage.

As a simple example of a radiosynthesis, for which a different procedure than that used for inactive materials is chosen, we cite the production of sodium cyanide by the reduction of barium carbonate with sodium azide[8].

$$NaN_3 + Ba\overset{*}{C}O_3 \rightarrow Na\overset{*}{C}N + N_2 + O_2 + BaO$$

An extreme example would be a case in which one starts with the complete, but unlabeled, compound, removes a part of the molecule, and replaces it with an identical, but labeled, moiety. Cholestenone-3-^{14}C and testosterone-3-^{14}C have been prepared by this procedure[9,10].

In the synthesis of tritium compounds the isotope is often added to double bonds by catalytic hydrogenation[11]. Oleic acid thus yields labeled stearic acid[12] which can, for many purposes, replace the much more costly carbon-labeled substance. Sterols have also been catalytically hydrogenated with tritium[12] (p. 303). The possibility of introducing tritium by catalyzed exchange will be discussed later.

In the *fourth* place, the decomposition of labeled compounds by their own radiation during storage must be taken into account (Chapter VI). The loss of labeled material is here less to be feared than the loss of radiochemical purity. In case of doubt it is well, therefore, to subject the substance before use to a purification according to the principles discussed in Chapter III.

Extensive compilations of radiosynthetic methods may be found in the literature, especially with reference to carbon, in which case procedures derived from conventional methods have been particularly emphasized [2,11,13–20,22,23]. As far as tritium is concerned, the extensive knowledge obtained in the synthesis of deuterium-labeled compounds is applicable [11,15,16,24]. A monograph on syntheses with radiosulfur is available[21].

2. Specific Radiochemical Syntheses

Besides the radiosyntheses derived from conventional procedures there are also some methods which can be designated as 'specifically radiochemical' and which have no counterpart in the chemistry of inactive materials.

In the *first* group of such syntheses, which may be termed exchange syntheses, the complete, but inactive, molecule is permitted, under suitable conditions, to exchange an atom or group of atoms with a radioactive molecule. An inactive atom is thus replaced by a chemically identical, but active, atom. The labeled molecule formed is useful for tracer experiments only if the radioactive atom is not lost by exchange during the experiments.

For example, it is useless to label alcohols and sugars in the hydroxyl hydrogen, or amines in the hydrogen of the amino group, since these dissociable hydrogen atoms are easily lost again by exchange with water (p. 17). Another example of an atom which is exchangeable even under ordinary conditions is the iodine of di-iodotyrosine[25].

On the other hand, carbon compounds do not under normal conditions exchange their carbon, nor do methyl or (most) methylene groups exchange their hydrogen, thio-compounds and H_2S their sulfur[26] or phosphoric acid and its organic esters their phosphorus[27–30] (Section X, *2*).

It is sometimes possible to perform a radiosynthesis by forcing an exchange under special conditions, while the exchange in the reverse direction does not take place under the conditions of the experiment. Such exchanges may be brought about by elevated temperature or by a suitable catalyst[11,31].

At high temperatures, for example, saturated fatty acids treated with heavy sulfuric acid yield α-deutero fatty acids[32]. In the presence of a platinum catalyst the purine ring can be labeled with radiohydrogen; in the absence of the catalyst no reverse exchange occurs, not even on treatment with acid or alkali[33]. Sterols[34,35] and dimethylbenzanthracene[36] are also labeled with hydrogen using platinum as a catalyst.

A method for preparing labeled acetic acid is also worth noting. Malonic acid exchanges all its hydrogen atoms with labeled water. It is then thermally decarboxylated, and acetic acid is formed, the methyl group of which no longer exchanges its hydrogen[37].

$$CH_2(COOH)_2 + THO \rightarrow CT_2(COOT)_2 \rightarrow CO_2 + CT_3COOT$$

For the transfer of ^{35}S from hydrogen sulfide to cysteine, enzymes can be used as catalysts[38]. Such methods can actually be regarded as biosyntheses (*cf.* below). Methods for the production of labeled organic phosphates by enzymatic exchange will be discussed on p. 162. Radiosyntheses by enzymatic exchange can be carried out with carbon compounds as well. An example is the preparation of sucrose labeled only in the fructose moiety, from active fructose and inactive sucrose, using an extract of *Pseudomonas saccharophila*[39–41]. Further examples can be deduced from the enzyme-catalyzed exchanges of section X, *2*. Radiocarbon can even be introduced into C–C bonds by enzymatic exchange procedures.

A *second* group of specifically radiochemical syntheses utilizes the energy released in the transmutation of the atomic nucleus to force the entrance of the radioactive atom into particular molecules. (In connection with the discussion of the Szilard effect, p. 13, it has already been pointed out that this energy can cause chemical changes). The radioatom in *statu nascendi* thus penetrates a neighboring molecule, where it is bound, and labels the molecule.

Neutron bombardment of halogenated hydrocarbons produces halogen-labeled substituted hydrocarbons with one less hydrogen atom, *e.g.*, $C\overset{*}{B}rBr_3$ from $CHBr_3$. Suitable mixtures can also be used as reactants; CBr_4 dissolved in benzene, for example, yields among other products $C_6H_5\overset{*}{B}r$[42, 43]. When radiocarbon is produced in a pile by bombardment of nitrogen with slow neutrons (p. 11), a variety of labeled carbon compounds, depending upon the kind of nitrogen compounds used, is obtained[44]. For example, dry urea, hydrazine, or glycine yield up to half of the carbon in the form of hydrogen cyanide, but irradiation of solutions of these compounds produces no active HCN[45].

Similar investigations have been made of other nitrogen compounds [46–51]. The preparation of ^{14}C-nicotinic acid by irradiation of nicotinamide with neutrons has proved practicable[52] (p. 243). The production of ^{14}C-paraffins by the bombardment of mixtures of aniline and paraffins is also feasible[53–55]. A review of this procedure for 'labeling by recoil' has appeared[56]. Finally, organic molecules (like benzene) have been labeled by bombardment with a current of ions containing radiocarbon; since the energy of the beam can be kept low, it is possible to avoid extensive decomposition of the target[57–60].

Tritium ions of high velocity, produced for example by the capture of slow neutrons in a pile by lithium (p. 11), also penetrate molecules and label them[61, 62].

Such substitution has been observed on irradiation of mixtures of lithium salts with sugars[63], reserpine[64], benzoic acid[63], nicotinic acid[65] and *p*-aminosalicylic acid[66]. It is advantageous to employ beams of ions also when using tritium[67].

An appreciable amount of labeling occurs when organic compounds are merely kept in close contact with tritium gas[68, 69]. Apparently products of radiation-decomposition of the 'substrate' react with the tritium. Labeling can be enhanced by application of an external γ-ray source[70] or of an electric discharge[67]. Radioactive digitoxin[71, 72], proteins[73] and aminosalicylic acid[74], for example, have been made by this method of 'radiation labeling'. It was later found that ^{14}C can likewise be introduced into molecules by storage under radioactive CO_2 or C_2H_2 provided an additional source of radiation, *e.g.* ^{85}Kr, is present[75, 76].

After recoil labeling or radiation labeling, a rigorous radiochemical separation from radioactive contaminants (by-products) is needed[77, 78]. Another disadvantage is the uncertainty about the distribution of the radioactive atoms within the labeled molecules. This distribution is generally far from uniform.

In the *third* place, a possibility exists of directly activating a complete, but inactive, compound. This is done by a nuclear reaction, especially by the capture of slow neutrons. In many cases the cations of inorganic salts are activated in this manner in piles; for example, radioactive cobalt sulfate or gold chloride can be produced. The radiocobalt or -gold is then of course exchangeable, in contrast to covalently-bound or complexed atoms.

When a covalent or complex compound is rendered radioactive by direct irradiation, it is necessary to be sure that a Szilard effect does not occur, *i.e.*, that the newly-formed radioatom is not lost from the molecule. An example of this effect was found in the attempts to activate the cobalt atom of vitamin B_{12} by neutron capture[79, 80]; subsequent investigation disclosed that almost all of the radiocobalt had been expelled from the molecules of the vitamin[81, 82].

In the *fourth* place, we note the possibility of labeling macromolecules by the introduction of a small amount of a foreign atom. The most important example is the labeling of proteins, such as serum albumin[83–85] or insulin[86], with radioiodine[87]. Such compounds are employed on the assumption (which must of course be tested in each individual case) that minor substitutions will not appreciably alter the biochemical behavior of the material. The situation is complicated by the fact that the quantities of iodine taken up by the individual molecules are inevitably not the same, so that the labeled material is not homogeneous (Section XVI,*1*).

3. Biosyntheses

More or less complicated substances which occur in living beings can often be obtained in labeled form by so-called biosynthesis, carried out *in vivo* or *in vitro*. (Radiosynthetic procedures which do not employ living matter or enzymes may be designated as 'abiosynthetic'.) Relatively simple labeled substances are added to the living matter or to preparations therefrom, and the synthesis is brought about by the enzymes. Many radiosynthetic procedures with higher plants and animals as well as with microbes have been described. Reviews may be found in the literature[15, 18, 20, 88].

Radiobiosyntheses are carried out on a large scale in connection with atomic energy laboratories. At the Argonne National Laboratory in Chicago, for example, labeled compounds for biological and agricultural research are prepared in sealed rooms of 10,000 liters' capacity, in which tobacco, alfalfa, rye, onions, artichokes, tradescantia, digitalis, poppy, rubber, soy and buckwheat are grown. Besides radiocarbon, radiophosphorus, -iron, -sulfur, and -zinc can be introduced. The distribution of the radioelements within the plants can be controlled by the conditions of cultivation[89] (p. 168).

Biosynthesis under normal conditions frequently leads to the formation of uniformly-labeled compounds. This is especially true of radiophotosynthesis, as for example in the production of labeled sugars by algae or green leaves. Some examples to be given later on, however, show that in other cases the labeling is strongly selective. Biosynthesis yields the naturally-occurring optical isomers of compounds with asymmetric carbon atoms.

Many radiophotosyntheses have been carried out, and many procedures and experimental set-ups have been described[90–93]. Labeled sugars are formed, for example, by the action of radioactive CO_2 on illuminated leaves. When assimilation is complete, the sugars are extracted with aqueous alcohol, acids and bases are removed with ion exchangers, and the sugars are separated from one another by paper chromatography (p. 22). It is not necessary to add inactive sugars as carriers, and so compounds with extremely high specific activities are obtained; *e.g.*, $\overset{*}{C}O_2$ with 12% isotopic purity yields sugars of 6% isotopic purity, corresponding to 300 million disintegrations per mg per minute (135 microcuries/mg)[94].

Other procedures produce plant pigments, such as chlorophyll (p. 296) or leaf carotenoids (p. 298), and alkaloids (p. 243). Labeled cellulose has been formed from radioglucose in cotton plants (p. 176). Labeled amino acids are obtained on a large scale by radiophotosynthesis with *Chlorella*[95].

The use of radiobiosynthesis is often indispensable. For instance, proteins with labeled carbon can be produced only biologically. As an example of a labeled protein (formed by a diseased plant) we may mention the tobacco mosaic virus (TMV).

TMV with labeled phosphorus was produced by growing the virus in the presence of radioactive phosphate[30, 96, 97]. TMV labeled in the carbon was obtained by photosynthesis in a tobacco leaf infected with the virus. The TMV was extracted from the leaf with buffer and purified by repeated isoelectric precipitations. It was found that the specific activity of the virus obtained in this way was the greater, within certain limits, the shorter the time which had elapsed between the infection of the leaf and the radiophotosynthesis. Apparently the 'younger' the virus is in the leaf the more avidly it takes up carbon dioxide[98, 99].

When carrying out the radiosynthesis of TMV it is therefore important to work with a 'young' virus, if high specific activity is needed. If a high total activity is required, on the other hand, it is better to work with a somewhat older virus. In this case, the specific activity is indeed lower, but this is more than compensated by the increase in the total quantity of TMV. It should also be noted that the distribution of radiocarbon within the virus molecule depends upon the age of the virus[100].

Biosyntheses with animals have also been described. However, much smaller yields are usually obtained than with plants, *i.e.*, a smaller fraction of the radioelement introduced is recovered in the form of the desired compounds. As examples of useful procedures we mention the synthesis of hemoglobin with labeled iron by feeding or injecting radioactive iron salts[101–103], the synthesis of ^{131}I-thyroxin[104], of ^{14}C-ATP[105], of β, γ-^{32}P-ATP[106] and of β-^{32}P-ATP[107] and γ-^{32}P-ATP[107]. (With regard to other enzymatic procedures for making ^{32}P-ATP see p. 162; an abiosynthetic method has also been described[108]). Influenza virus with labeled phosphorus has been grown in hen's eggs[109, 110].

Plasma proteins have been labeled by feeding labeled algal proteins[111] (Chapter XVI), and cholesterol by injecting labeled acetic acid. In the case of cholesterol it was found that certain carbon atoms of the cholesterol acquired the label only from the carboxyl carbon of the acetate, and others only from the methyl carbon (Chapter XVI). Labeled glycogen has been prepared biosynthetically in intact rats[112], in rat liver slices[113] and in *Drosophila*[114].

Finally, it may be advantageous to use micro-organisms for radiobiosynthesis. Examples are the formation of labeled DPN and TPN by *Lactobacillus plantarum*[115] and of labeled acetic acid by fermentation of glucose with *Clostridium thermoaceticum*[116, 117] or by reduction of CO_2 with *Clostridium aceticum*[117]. Labeled cellulose has been obtained from mannitol or glucose by the use of *Acetobacter xylinum* (p. 176). Labeled amino acids, purines and pyrimidines are formed by growing yeast on radioactive sugar[90]. Radiophotosynthesis with *Chlorella* has already been mentioned.

When lactate is fermented by *Butyribacterium rettgeri*, some butyric acid is produced by way of acetic acid as an intermediate. If specifically-labeled acetic acid is introduced into the medium, specifically-labeled butyric acid is obtained[117, 118] *viz.*

Substrate	Product
$CH_3\overset{*}{C}OOH$	$CH_3\overset{*}{C}H_2CH_2\overset{*}{C}OOH$
$\overset{*}{C}H_3COOH$	$\overset{*}{C}H_3CH_2\overset{*}{C}H_2COOH$
$\overset{*}{C}H_3\overset{*}{C}OOH$	$\overset{*}{C}H_3\overset{*}{C}H_2\overset{*}{C}H_2\overset{*}{C}OOH$

Streptomycin with labeled carbon has been made by growing *Streptomyces griseus* on a medium containing radioactive starch, glucose, or amino acids[119, 120]. Penicillin with labeled sulfur (from sulfur-containing media[121, 122]; p. 142) or carbon[123], carbon-labeled terramycin[124] and pneumococcal polysaccharides (see p. 283 for immunological experiments)[125] as well as vitamin B_{12} with radiocobalt[126–129] have also been described.

The synthetic capabilities of living systems, which can often be utilized for preparative purposes, are more fully discussed in Chapters XI–XVI.

Finally, physiological entities which consist of many molecules, such as bacteria[130–136], red blood cells[101, 137, 138], tumor cells[139, 140], mammalian sperm[141–145] and even whole animals[146–148] can be labeled by biosynthetic methods.

4. Double Labeling

For certain biochemical investigations it is advisable to use doubly-labeled compounds, *i.e.* compounds labeled with two different radioactive atoms.

The double-labeling can be done with two different radioisotopes of the same element in such a way that some of the molecules contain one radioisotope, and other, chemically identical, molecules the other radioisotope, but with the radioactive atom always in the same position in the molecule. When the two radioisotopes have different half-lives, a composite decay curve is obtained which falls off more slowly than that of the shorter-lived isotope. This can be an advantage in the investigation of processes which require long periods of time.

Since the results of the measurement with a given apparatus depend upon the nature of the radiations and hence, of the nuclides, and the ratio of the quantities of the two isotopes changes with time, the activity of any sample, at any given time, must be compared to that of a reference sample, which contains the same mixture of isotopes and which was set aside prior to the experiment. The difference in the half-lives (2.9 years and 45 days), and also in the properties of the radiations, is particularly marked in the case of the two isotopes of iron, ^{55}Fe and ^{59}Fe (see also p. 14).

In other types of experiments, advantage can be taken of the difference in the radiations. It may be of interest, for example, to follow the fate of two portions of a given salt of iron which enter the body by two different pathways[149] or at two different times. The procedure is the same if one wishes to determine the fate of two different iron compounds; these may be administered simultaneously or at different times. Likewise the metabolism of donor blood can be followed independently of that of the animals' own blood. In all these cases one substance is labeled with one isotope, and the other with the other isotope, and the isotopes are distinguished by the difference between their rays. The samples are therefore measured with two different kinds of instruments, each of which detects primarily or exclusively the rays of one isotope[150–152].

Double labeling with two different radioelements can also be utilized in biological research. The pairs ^{14}C + ^{32}P or ^{32}P + ^{42}K have been administered simultaneously to higher plants to study translocation[153, 154] (Chapter X). The metabolism of cattle has been investigated with ^{45}Ca + ^{32}P[155] (Chapter X), and the synthesis of nucleic acids by rats[156] with ^{14}C + ^{32}P (Chapter XV).

Single compounds can be labeled, in different positions of the molecules, either with different isotopes of the same element or with different radioelements. It is possible by use of double labeling to determine whether or not a group of atoms is completely transferred from one molecule to another. Liver slices, for example, were incubated with isovaleric acid-4,4′-^{13}C-1-^{14}C, various metabolic products (ketones, fatty acids, cholesterol) were isolated and the ^{14}C/^{13}C ratio determined. If this ratio was different from that in the parent substance, the carbon chain must have been cleaved[157].

Tobacco mosaic virus (see also the preceding section) was labeled with ^{14}C and ^{32}P. The constancy of the ratio of the two activities after various physiological processes indicated that the particles remained intact[158].

Methyl groups were labeled both in the carbon and in the hydrogen in order to investigate their transfer between different compounds in tissues[159–164]. If the process occurs without destruction of the methyl groups (transmethylation), the ratio of isotopic carbon and hydrogen must be the same before and after transfer. But if the methyl group is first degraded with the formation of oxygen-containing one-carbon compounds, and the carbon utilized subsequently to rebuild methyl groups, no such constancy of the ratio is to be expected.

In this connection one important source of error is to be guarded against[165, 166]. In the usual radiosynthesis, in which a relatively small number of isotopic atoms is randomly distributed among a large number of molecules of labeled compound, a mixture of relatively few carbon-labeled and relatively few hydrogen-labeled molecules is obtained, along with a great excess of unlabeled molecules. One may in this case speak of 'intermolecular' double labeling. The number of individual doubly-labeled molecules is very small because of the small probability that both radioactive atoms will enter a given molecule.

The two types of labeled molecules are subject to isotope effects (see below) of different magnitudes. Since isotope effects are appreciable in the case of hydrogen, a deviation in the 'isotope ratio' during transmethylation is to be expected on this basis alone. Such isotope effects have been demonstrated by comparison of the behavior of CH_3-, CD_3-, CH_2D-, and CH_2T-groups, and found to be quite important[165, 167–169].

It is possible to guard against this isotope effect by actually putting both labels into the same molecule ('intramolecular' double-labeling). This is not practicable for $^{14}C + {}^{3}H$, since neither isotope is commercially available in the pure state, and the labeled compound will therefore not be uniform, but must contain the combinations $^{14}C + {}^{1}H$ and $^{12}C + {}^{3}H$ in large amounts. It is, however, possible to produce unobjectionable intramolecular double labeling with the combination $^{14}C + {}^{2}H$, since heavy hydrogen in nearly pure form can be obtained. The combination $^{12}C + {}^{2}H$ will indeed occur as well, but does not do much harm, since the isotope effect with carbon is unimportant. It has been demonstrated in this way that in rats transmethylation from methionine to choline and creatine does occur[170]. On the other hand, formaldehyde (labeled with ^{14}C and D) is not the immediate precursor of the labile methyl groups of creatine or choline when injected into rats[171].

In double labeling with heavy atoms, where isotope effects are less marked, the objections cited do not apply, particularly when the labeled molecules are very large and react as complete entities. Proteins doubly-labeled with radioiodine and radiocarbon will be discussed in Section XVI, *1.*

5. Selective Degradation of Labeled Compounds

Both in the testing of substances prepared by radiosynthesis and in the examination of compounds obtained from biological material in metabolic experiments, it is important to determine which positions in the molecule are radioactive. Organic molecules with labeled carbon atoms must therefore be subjected to a clear-cut, stepwise degradation. The simple degradation products obtained—which in the limiting case may contain only one carbon atom each—are examined individually for their activity;

it is then possible to construct a sort of 'activity map' of the original molecule. Similar procedures are necessary in the cases of hydrogen, sulfur, etc.

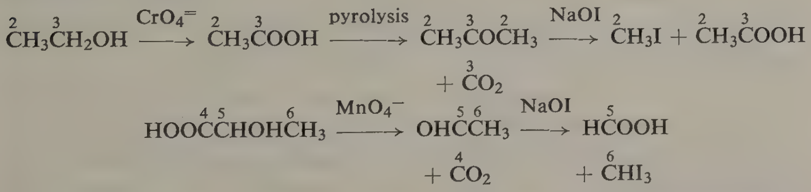
Several authors[113, 172–174] have described methods of degrading carbohydrates so that the activity of each carbon atom can be determined separately. In one method for the determination of the distribution of activity in glucose, methyl glucoside is first oxidized with periodic acid, and the products subjected to further degradation. The activity of carbon atoms 2–5 can then be measured separately, and the activity of C-1 determined by the difference from the total activity of the glucose[175].

Specific degradation can also be easily achieved by applying suitable microörganisms. For the degradation of glucose, *Lactobacillus casei* is used. The lactic acid formed is treated with permanganate to yield acetaldehyde and CO_2 (from the carboxyl group: C-3 and C-4 of the glucose). When the acetaldehyde is degraded by the iodoform reaction, the carbon of the iodoform corresponds to C-1 and C-6 of the glucose molecule, and that of the formic acid to C-2 and C-5. This method, then, gives the activity of pairs of atoms[172, 174].

Even more definite results are obtained by another microbiological procedure. *Leuconostoc mesenteroides* degrades glucose according to the equation[176–179]:

$$C_6H_{12}O_6 \rightarrow \overset{1}{C}O_2 + \overset{2}{C}H_3\overset{3}{C}H_2OH + HOO\overset{4}{C}\overset{5}{C}HOH\overset{6}{C}H_3$$

The lactic acid and ethanol are degraded separately by the following chemical procedures:



$$\overset{2}{C}H_3\overset{3}{C}H_2OH \xrightarrow{CrO_4^{=}} \overset{2}{C}H_3\overset{3}{C}OOH \xrightarrow{\text{pyrolysis}} \underset{+\,\overset{3}{C}O_2}{\overset{2}{C}H_3\overset{3}{C}O\overset{2}{C}H_3} \xrightarrow{NaOI} \overset{2}{C}H_3I + \overset{2}{C}H_3\overset{3}{C}OOH$$

$$HOO\overset{4}{C}\overset{5}{C}HOH\overset{6}{C}H_3 \xrightarrow{MnO_4^{-}} \underset{+\,\overset{4}{C}O_2}{OH\overset{5}{C}\overset{6}{C}H_3} \xrightarrow{NaOI} \underset{+\,\overset{6}{C}HI_3}{H\overset{5}{C}OOH}$$

Finally the activities of the individual carbon atoms are determined.

Microbiological methods for the assay of the radioactivity of desoxyribose have also been reported[180, 181].

Other degradative procedures have been described for other purposes. Some of them will be mentioned in connection with the analysis of fats (p. 212), purines (p. 255), pyrimidines (p. 259), porphyrins (p. 290) and sterols (p. 297). A comprehensive collection of degradation methods has been published[182].

The interesting problem arises occasionally whether several positions in one and the same individual molecule are labeled. This question obviously cannot be answered by measurements of the activities of the products obtained by specific degradation, since it is not known to what extent the labeled carbon atoms in the various degradation products stem from the same molecules.

Such a problem existed during the investigation of the formation of acetic acid by *Clostridium thermoaceticum* in the presence of glucose and carbon dioxide. According to one mechanism proposed[116] the acetic acid arises by two different, successive reactions (*cf.* p. 37):

$$\begin{array}{ll} (a) & C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 8[H] + 2CO_2 \\ (b) & 8[H] + 2CO_2 \rightarrow CH_3COOH + 2H_2O \end{array}$$

If the fermentation takes place in an atmosphere of labeled CO_2, the acetic acid formed by reaction (*a*) would not be labeled at all, while that formed by reaction (*b*) would be labeled in both carbon atoms. On the other hand, molecules arising by condensation of a molecule of CO_2 with an unlabeled partner by some other reaction would be labeled in only one of the carbon atoms.

To decide this point, carbon dioxide with a high content of ^{13}C was introduced, the acetic acid reduced to ethylene and examined with a mass spectrometer. The appearance of ions with a mass of 30—to a greater extent than could be explained on the basis of the natural occurrence of ^{13}C—demonstrated that in at least some cases both carbon atoms of one and the same acetic acid molecule were derived from the CO_2, as demanded by the above scheme[183].

In a similar way one can decide whether a symmetrical molecule is labeled in all equivalent positions. For instance, some of the succinic acid molecules formed by 'head-to-head' condensation of two molecules of $\overset{*}{C}H_3COOH$ must be labeled in both methylene groups.

On the other hand, succinic acid molecules formed in the citric acid cycle from unlabeled oxalacetate and methyl-labeled acetic acid (p. 193) can be labeled in one methylene group only. Since the methylene groups are chemically identical, they can not be separated by specific degradation and investigated individually. A decision as to whether both groups of a single molecule are labeled can be made by decarboxylation and dehydrogenation to ethylene, followed by mass spectrometry[184].

The last-named problems are among the few which can indeed be solved by isotopic methods but not by radiochemistry alone[185,186]. Here it is necessary not only to determine the content of isotope, but also to separate the labeled molecules from the rest. It is certainly conceivable that radioactive labels may be used, and—after mass spectrometric separation—the fraction of doubly-labeled molecules determined by their activity. However, radiation damage would probably be prohibitive if the number of doubly-labeled molecules were not small.

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CHAPTER V

ISOTOPE EFFECTS

1. General Considerations

The difference in the chemical behavior of isotopes, both in biochemistry and in other fields of chemistry, can usually be neglected. There are special cases, however, in which the difference must be taken into account. The 'isotope effect' may interfere (p. 40), or it may, on the other hand, make possible definite conclusions as to reaction mechanisms. Reviews on isotope effects have appeared in the literature[1–9].

Since the electronic shells of isotopes are alike, the isotope effects always depend upon the masses of the atoms, and increase in importance with the ratio of the masses. The magnitude of the isotope effects is not influenced by the radioactivity of the atom. Up to the moment of disintegration, radioatoms behave exactly as do stable atoms of the same mass.

The difference between isotopes can lead to differences in equilibria and to differences in reaction velocities. Isotope effects can be calculated, if certain assumptions are made.

The greatest differences are to be expected between the atoms of hydrogen[10] (mass ratio protium : deuterium : tritium = 1 : 2 : 3). With heavy atoms, on the other hand, the ratio of the masses differs but little from unity; isotope effects are insignificant in such cases.

When entire molecules or large groups of atoms are involved, as is often the case in organic and biological chemistry, the isotope effects are less prominent than in reactions involving atoms. The difference in mass between the isotopic atoms is then effectively reduced by the presence of other atoms bound to them. This diminution of the isotope effect is particularly marked when the isotopic atoms are far removed from the site of reaction.

The poisonous nature of highly concentrated deuterium (in the form of heavy water, for example) must, in the last analysis, also be regarded as an isotope effect. Algae are damaged by heavy water in concentrations of more than 30%[11–14], and tobacco seeds do not germinate in water which contains more than 40% heavy water[15]. Extensive histological studies on mice have shown that heavy water behaves, in some ways, like a radiomimetic substance[13, 14, 16, 17].

2. Equilibrium Effects

As an example of the shifting of a chemical equilibrium by isotope effects, we may note that the experimentally determined distribution coefficient of deuterium between water and gaseous hydrogen at 25° has the large value of 3.7, and that of tritium is as much as 6.3[18]. The heavy isotope is therefore concentrated in the liquid water by the reaction $H_2O + HD \rightleftharpoons HDO + H_2$. The distribution coefficient of deuterium between ethyl mercaptan and water is 0.43[19], between aniline and water, 1.11[20]. Deuterium is enriched by a factor of 3.83 in the carboxyl group of acetic acid in equilibrium with hydrogen gas; tritium, by a factor of 6.86[21].

In the case of the carbon isotopes 12, 13, and 14, the corresponding isotope effects are of course much less important. Enrichments and impoverishments of only a few percent have been observed. A relatively large effect was noted in the equilibrium:

$$[Co(NH_3)_4]^{12}CO_3^+ + {}^{14}CO_3^= \rightleftharpoons [Co(NH_3)_4]^{14}CO_3^+ + {}^{12}CO_3^=$$

The equilibrium constant is 0.89[22–24], but a reaction of this sort has no biochemical significance.

The effect of isotopes on equilibria depends upon the temperature. It is thus possible, from the position of a 'frozen' equilibrium, to calculate the temperature which prevailed at the time the equilibrium was established. Urey[5, 6, 25] bases his procedure for measuring 'paleotemperatures' on the determination of the ^{18}O content in the calcium carbonate of marine animals of earlier geological epochs in comparison with the content of this isotope in (recent) sea-water; in other words the equilibrium

$$CaC^{16}O_2{}^{18}O + H_2{}^{16}O \rightleftharpoons CaC^{16}O_3 + H_2{}^{18}O$$

is determined.

3. Kinetic Effects

Isotope effects on reaction velocities ('kinetic isotope effects') are often greater than those on equilibria. These effects are also more important to the biochemist, since biochemical reactions do not usually lead to the establishment of equilibria. The magnitude of the effect is expressed by the quotient of the reaction rates with the two isotopes, k_1/k_2 $(= K)$. The enrichment or impoverishment with respect to one or the other of the isotopes attains the value K only at the beginning of the reaction; after the reaction has run for a time, the reactant loses the faster-reacting isotope, and the ratio of the reaction rates drops. In general, the lighter isotope reacts more rapidly than the heavier one.

Theoretical maximum values for kinetic isotope effects have been computed[26–28], but in the reactions observed to date the maximum values have never even been approached. The theory also predicts that the quantity $(K-1)$ will increase almost linearly with the mass. For example, it should be twice as great for ^3H (T) as for ^2H (D), and twice as great for ^{14}C as for ^{13}C, when ^1H and ^{12}C are taken as the standards. Experiments have confirmed these predictions in some cases (acid hydrolysis of urea; see below), but not in others[3, 5].

The largest kinetic isotope effects occur, of course, with hydrogen. In the alkaline hydrolysis of tripropylsilane-T, for example, the isotope effect k_T/k_H is 0.80[29]; and that for the bromine oxidation of the tritium-labeled methylene group of ethanol to acetaldehyde is 0.57[30]. Strong isotope effects were likewise obtained in the separation of D- or T-labeled hydrocarbons by gas–liquid chromatography[31].

A complicated biological system was involved when rats were fed with water labeled both with D and with T; the fatty acids of the liver showed an increase of 18% in the D/T ratio, and the glycogen an increase of 8%[32–34]. Similar results on intact animals and plants have been reported from other laboratories[35–37]; the experiments are difficult to interpret, but they do indicate the necessity for using caution in the evaluation of results obtained with isotopic hydrogen. Some microörganisms produce molecular hydrogen strongly enriched in protium[38].

In the case of carbon, kinetic isotope effects between ^{12}C and ^{14}C up to $K=1.5$ have occasionally been reported, but the really reliable results on well-defined systems are much lower, usually falling below 1.2. The isotope effect of the much-investigated thermal decarboxylation of ^{14}C-labeled malonic acid, for example, appears to exceed unity by only a few percent; the specific activity of the acetic acid produced exceeds that of the carbon dioxide by only this amount[1, 2, 6].

Another example is the decomposition of oxalic acid with sulfuric acid to form CO_2 and CO; the CO_2 is enriched in ^{13}C relative to ^{12}C by a factor of 1.033[39]. When urea is hydrolyzed by acid, the CO_2 is poorer in ^{13}C by a factor of 1.055, and in ^{14}C by a factor of 1.10[40, 41]. When amino acids are separated by exchange resins according to the procedure of Moore and Stein, the speed of appearance of labeled acids in the eluate is a little different from that of the inactive acids. The maxima of the color reactions do not, therefore, exactly coincide with the maxima of radioactivity[42].

In photosynthesis with barley seedlings and algae, ^{14}C is reported to be taken up 15–17% more slowly than ^{12}C[43, 44]. Some other authors have, however, criticized these results[6, 45], or observed smaller effects with various types of plants[5, 46, 47]. In any case, it has been shown specifically that there is no qualitative difference between the assimilation of stable and of radioactive carbon[48].

In order to explain abnormally large fractionation of the isotopes during photosynthesis, the proposal has been made that the enrichment is due to multi-stage assimilation. Part of the freshly-assimilated carbon is supposedly respired again, part of the respired carbon is re-assimilated, etc. This hypothesis also claims to explain certain differences in the $^{13}C/^{12}C$ ratios between plants grown under different climatic conditions [6, 49–53].

In contrast to the equilibrium effects, the kinetic effects can depend upon the reaction mechanism, so that the enrichment of isotopes can vary according to the reaction path[3]. For example, the enzymatic hydrolysis of carbon-labeled urea proceeds with reaction velocity ratios of 1.010 and 1.032 for $^{12}C/^{13}C$ and $^{12}C/^{14}C$, respectively, while the acid hydrolysis exhibits corresponding ratios of 1.055 and 1.10[41, 54]. Similarly, the ratios of the rates of oxidation of HCOONa and DCOONa depend strongly on whether the oxidation is carried out with permanganate or with H_2O_2 in presence of catalase[55, 55a, 55b].

When sulfate is reduced by bacteria, the light isotopes of sulfur accumulate in the hydrogen sulfide, in organic sulfur, and in the elementary sulfur formed therefrom by oxidation, so that it is possible under certain circumstances to distinguish between sulfur deposits formed microbiologically and by inorganic processes[3, 6].

It does not seem impossible, therefore, to draw conclusions about reaction mechanisms from the observed ratios of isotopes—*e.g.* in living matter. It is of course necessary to prevent the reactions reaching equilibrium, since the effect of the reaction path is then eliminated; but, as has already been pointed out, this requirement is very frequently fulfilled in living systems.

The possibility of kinetic isotope effects is also to be considered in the combustion of radioactive hydrogen or carbon samples for the measurement of the activity. If the combustion is incomplete, the products may have a different isotopic composition from the starting material. It has been observed, for example, that heavy acetaldehyde CH_3CDO is more slowly oxidized by chromic acid or bromine than is light acetaldehyde CH_3CHO[56–58]. Incomplete absorption by the alkali of the CO_2 formed by combustion should also to avoided, since a kinetic isotope effect can occur here as well; the absorption of $^{12}CO_2$ is 1.4% faster than that of $^{13}CO_2$[59].

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CHAPTER VI

RADIATION CHEMISTRY

Radiation chemistry has in recent years experienced a rapid expansion [1–17]. For reasons discussed on p. 5 it is here possible only to refer briefly to the chemical effects of radiation on that type of chemical system which is biochemically most important, namely, aqueous solutions.

When radioactive rays penetrate matter they lose their energy by the excitation and ionization of molecules. The radiation dose due to electromagnetic (X- or γ-) rays is measured in 'roentgens' (r). The exact definition of the roentgen cannot be discussed here [18]; as an example we may note that a dose of 1 r corresponds to the absorption of 83 ergs per g of air or 93 ergs per g of water (or tissue which is mostly water). For a dose which causes the absorption of 100 ergs/g in any substance, the term 'rad' has been coined. This definition is no longer limited to electromagnetic radiation; the dose supplied by fast particles (α- or β-rays) can also be expressed directly in rads. Literature on the measurement of doses will be given on p. 70.

In aqueous solution the greater part of the energy is absorbed by the water itself. According to Weiss [19–21] the predominant primary process is the loss of an electron by a water molecule, which changes into a positive water ion. The electron is immediately taken up by another molecule of water, to give a negative water ion. Both water ions are unstable and decompose spontaneously:

$$H_2O^+ \rightarrow H^+ + OH$$
$$H_2O^- \rightarrow H + OH^-$$

The occurrence of other reactions in water is probable [22, 23].

The two ions, which arise by these two reactions, are chemically unimportant, since they are not plentiful enough to change the local pH-value; moreover, they quickly recombine to form water. The hydrogen atoms are also probably not very active in most systems of biochemical interest [22, 24]. If they do not react in other ways, the hydrogen atoms finally form molecular hydrogen. The hydroxyl radicals OH, on the other hand, have a strong oxidizing action and attack organic matter. Most of the unconsumed hydroxyl radicals combine in pairs to form H_2O_2. The short-lived oxidizing radical HO_2 is also formed in a variety of ways.

The yield in radiation-chemical reactions is often expressed as the *G*-value. This is the number of molecules produced per 100 electron volts. The decomposition reactions are not always well defined. Organic substances, for example benzene derivatives[25–28] and sterols[29–31], can be oxidized or dehydrogenated, hexoses degraded[32–34], sugar alcohols converted to hexoses[32, 34], and lactones to reductones[35]. Irradiation has been proposed as a preparative method[36, 37]. The occasional specificity of the effects is illustrated by the fact that even small amounts of pyruvate can compete successfully with large amounts of lactate for the primary products of the decomposition of water[38].

The attack of the radicals on enzymes has been followed quantitatively. For example, solutions of carboxypeptidase are inactivated by radiation. The attack can to some extent be diverted by reducing substances, which act protectively, provided that they are present at the time of irradiation. Depending upon the conditions, doses of 10^2–10^4 r are required for the partial inactivation of enzymes in solution[39–42].

In concentrated solutions of organic materials the 'indirect' action of the decomposition products of water, which has just been described, is accompanied by direct action, since the probability is now increased that the organic molecules themselves will be struck by the rays. These direct actions are also important in the radiation-biological damage, which is to be discussed in Chapter VII[43–46].

The auto-decomposition of labeled compounds, which occurs on storage (p. 32), is a radiation-chemical effect. The decomposition is especially marked when the substance is stored as a uniform, thick layer, since all the energy of the rays is then absorbed within the substance itself. Decomposition is decreased by storage in a thin layer, from which the radiation can escape, or in mixtures with inert substances (or in solution). Approximate calculations[47] indicate that under typical conditions (activity 1 millicurie, β-rays of average energy 5×10^5 eV) the decomposition in a thick layer may be of the order of 1/10 micromol of material per day. Of course, a micromol amounts to a large weight, if the molecular weight is large; one would therefore expect proteins and nucleic acids to show especially great sensitivity to radiation, if it is assumed that a small alteration of the molecule is sufficient to impair the characteristic properties of the material.

The decomposition has frequently been investigated experimentally, and has in some cases proved to be appreciably greater than was expected

[48–53]. A valuable survey of the auto-decomposition of pure, carbon-labeled organic compounds has appeared in the literature[54]. The distribution of the radiocarbon in the products of self-decomposition of glucose under various experimental conditions has been examined[55].

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CHAPTER VII

RADIATION BIOLOGY AND PROTECTION AGAINST RADIATION

1. General Considerations

Exaggerated notions of the dangers of work with radioactive substances are quite widespread. On the other hand, a too casual attitude is even less appropriate. It is necessary, therefore, for the biochemist who works with radioactive materials to be able to judge at least approximately whether he is working in the danger zone, and whether special precautions are indicated. For this purpose, a knowledge of the elements of radiation biology is essential.

We start with the assumption that radioactive rays, if they have any biological effect at all, can cause only harm. Despite the widespread opinion to the contrary, it appears to have never been established scientifically that the rays can cause anything but damage to human (or other) tissues; the beneficial effect often asserted to be produced by small doses has never been incontrovertibly established. However, in the case of plants, the alterations may under some circumstances be useful to man (for scientific purposes, agriculturally or esthetically).

Some interesting investigations have demonstrated that the growth of *Aspergillus niger* and also the beating of the isolated frog heart remain unaffected when the potassium of the nutrient medium (Ringer-solution) has only 1/60 as much of the radioactive isotope ^{40}K as normal, or when this isotope has 110 times the normal abundance[1,2]. It can be concluded that natural radioactivity has no major physiological effect as far as potassium and the given systems are concerned.

The biological effects of radiation are basically chemical, and involve radiation chemistry. Numerous texts[3–21] on radiation biology have appeared; only the fundamentals can be presented here.

The high biological effectiveness of radiation is most noteworthy[22–28]. A rough correlation exists between the effect of radiation and the place of the organism in the phylogenetic tree. Microbes are relatively insensitive, worms and insects are intermediate, and vertebrates are the most sensitive. The higher plants are less easily affected than the higher animals[7,29–38].

Just which radiation-chemical reactions give rise to the sensitivity which is evident in higher animals is still a matter of conjecture[21,39–44]. The effect on nucleic acids is probably more important than that on proteins. It is possible that the injury of even a few molecules of nucleic acid, if they are located at critical points, or the hindering of their synthesis, would cause much biological damage. Enzymatic systems, on the other hand, probably consist of large numbers of molecules and therefore become inactivated only by high doses of radiation.

To get some idea of the absolute value of the sensitivity to radiation, we note that radiation effects on intact vertebrates[45] or on vertebrate tissue in culture[46,47] have been observed already with doses as low as 10 r or less, but that they become obvious with living animals only at doses five times as great. When the whole human body is uniformly irradiated with X- or γ-rays and the radiation is delivered over a short period of time, the average fatal dose is approximately 400–500 r. This corresponds to an elevation of body temperature of only about 10^{-3} degrees.

Every tissue exhibits increased sensitivity when it is biologically active, *i.e.* when it is in the process of division. Germinating plants are more sensitive to radiation than air-dry plants[48]. On the other hand, certain reducing agents, especially those containing sulfhydryl groups, have a protective action, even *in vivo* [49–52]. Some review articles have dealt with the possibilities of reducing sensitivity to radiation[52–55].

2. Tolerance Doses—Effects of External Radiation

A tolerance dose of 0.3 r (300 milliroentgens) per week has been established for uniform whole-body irradiation with X- or γ-rays (*i.e.*, electromagnetic radiation) of persons who are exposed in the course of their work[56–61]. Recently, however, the further limitation has been imposed that the total dose per year should not exceed 5 r [62,63]. It is usually not permissible to expose oneself to higher dose rates for short periods, and to 'make it up' by periods of non-exposure.

The tolerance dose is determined on the basis of blood counts. Changes in blood counts are at present the most sensitive indication of radiation damage to an individual. In this sense, the bloodforming organs are the 'critical' ones. However, a still smaller tolerance has been assigned to the lens of the eye. In contrast to this, the hands may be exposed to 5 times the usual tolerance dose. It is assumed that no cumulative damage occurs, if the tolerance limit is observed, and that the healing processes keep pace with the damage.

β-rays are thought to be as effective as γ-rays. Lower tolerance doses apply, however, to more strongly ionizing rays. The biological effect of α-rays, per unit of energy absorbed, is reported to exceed that of β- or γ-rays by a factor of up to 20; this factor is termed the RBE (relative biological efficiency)[57–60, 62–68]. However, since the action of α- or β-rays is in practice always localized, and the RBE values depend on the organ, there is considerable uncertainty as to the tolerance dose.

Genetic damage by mutations is not considered when these tolerance doses are established. Such damage does not appear in the exposed individual, but only in his descendants. According to our present understanding of the subject, 'healing' of such damage does not occur at all, the mutations being irreversible. The number of mutations appears to be proportional to the total dose of radiation, and independent of the dose rate*. According to recent estimates, a dose of only 50 r is sufficient to double the spontaneous mutation rate in man; however, it is very doubtful whether one can with any confidence apply to man the results obtained with other animals[70].

In laying down the tolerance dose for external radiation, the carcinogenic effect is not considered either. The relation between dose and carcinogenesis is also not yet clear; in particular, it is not known whether or not a threshold value exists. The probability of the production of cancer is, however, very small in the range of doses which concerns the biochemist[71–77]. It is an interesting fact that plant tumors can also be induced by radiation[37].

Table 3 lists the radiation doses given by the γ-rays of several important radioelements, with and without lead shielding[78–82].

TABLE 3

DOSE RATES FROM SEVERAL γ-EMITTERS

(milliroentgen per mC per hour, at a distance of 1 meter)

Radioelement	*Thickness of lead shield (cm)*		
	0	2.54 (= 1 in.)	15.2 (= 6 in.)
^{24}Na	1.95	0.67	0.0032
^{60}Co	1.4	0.35	0.000027
^{131}I	0.23	0.025	–
^{198}Au	0.24	0.014	–
Radium and daughters	0.85	0.18	0.00032

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* Recently, doubt has been thrown on this assumption[69].

The highest activities employed in biochemical work are usually of the order of one millicurie. Therefore, the external effect of γ-rays can be a source of danger to the biochemist only in special cases, as in the receipt of shipments of large amounts of radioelements. External effects of α- or β-rays are in practice confined to the skin; however, since the rays are strongly absorbed by the walls of glass containers, by the air, etc., there is seldom any danger from the external effect of such rays.

3. Effects of Internal Radiation

The danger from so-called 'internal' effects of radiation is much more serious. When radioelements are taken into the body, they distribute themselves in the various organs in ways which depend upon their chemical nature (see Chapter X). They can remain there for very long periods of time, even for the whole life of the organism, and can exert their radiation effects at any point continuously if their half- life is sufficiently long. Because of their short ranges, the α- and β-rays are particularly important in this connection. Entrance into the body can occur by inhalation of vapors and dusts, or with food, drink, tobacco and cosmetics.

It is difficult to set tolerances for internal radiation effects [83–91]. The danger from each individual radioelement depends not only on its half-life and the type and energy of the radiations, but also upon its tendency to accumulate in an organ, and to take up a more or less dangerous position within that organ.

For example, the long-lived ^{22}Na is of course more serious than the short-lived ^{24}Na. Both isotopes, however, are relatively rapidly excreted in the urine; their so-called biological half-life is short. On the other hand, the elements Ca, Sr, Ra, Pb, and Pu are deposited in the bones and are never completely removed (Chapter X). There they can provoke bone tumors [77].

Experiments have demonstrated that plutonium is much more dangerous than the alkaline earths, when equal doses are considered and account is taken of the differences between the rays; whereas the alkaline earths (and also lead) are deposited in the mineral part of the bones, the plutonium—presumably in the form of a colloidal hydroxide or a basic salt—is deposited directly in the bone marrow, so that most of its α-rays are absorbed in this sensitive tissue.

For these reasons only rough estimates of the danger from various radioelements are possible. In Table 4 some of the important radioelements are listed according to the danger which they are presumed to present. In some cases the hazard has actually been determined by animal experiments[78]. Tables of 'short period tolerances for ingested radioelements' have also been compiled[92,93].

TABLE 4

RELATIVE DANGER OF RADIOELEMENTS (INTERNAL EFFECTS)

Exceptionally poisonous:	^{90}Sr + ^{90}Y, ^{210}Pb + ^{210}Bi [Ra (D + E)], ^{211}At, ^{210}Po, ^{226}Ra, ^{227}Ac, ^{233}U, ^{239}Pu, ^{241}Am, ^{242}Cm.
Very poisonous:	^{45}Ca, ^{59}Fe, ^{89}Sr, ^{91}Y, ^{106}Ru + ^{106}Rh, ^{131}I, ^{140}Ba + ^{140}La, ^{144}Ce + ^{144}Pr, ^{151}Sm, ^{154}Eu, ^{170}Tm, ^{234}Th + ^{234}Pa, Th (natural), U (natural).
Moderately poisonous:	^{24}Na, ^{32}P, ^{35}S, ^{36}Cl, ^{42}K, ^{46}Sc, ^{47}Sc, ^{48}Sc, ^{48}V, ^{56}Mn, ^{55}Fe, ^{60}Co, ^{59}Ni, ^{64}Cu, ^{65}Zn, ^{72}Ga, ^{76}As, ^{86}Rb, ^{95}Zr + ^{95}Nb, ^{95}Nb, ^{99}Mo, ^{96}Tc, ^{105}Rh, ^{103}Pd + ^{103}Rh, ^{105}Ag, ^{111}Ag, ^{109}Cd + ^{109}Ag, ^{113}Sn, ^{127}Te, ^{129}Te, ^{137}Cs + ^{137}Ba, ^{140}La, ^{143}Pr, ^{147}Pm, ^{166}Ho, ^{177}Lu, ^{182}Ta, ^{181}W, ^{183}Re, ^{190}Ir, ^{192}Ir, ^{191}Pt, ^{193}Pt, ^{196}Au, ^{198}Au, ^{199}Au, ^{200}Tl, ^{202}Tl, ^{204}Tl, ^{203}Pb.
Slightly poisonous:	^{3}H, ^{7}Be, ^{14}C, ^{18}F, ^{51}Cr, ^{71}Ge, ^{201}Tl.

In practice, however, the danger depends upon many factors. One factor is the specific activity. For example, radioiodine—a dangerous radioelement[94,95]—can accumulate in appreciable amounts in the thyroid gland only when its specific activity is high, because the organ has a high affinity for iodine, but only a small capacity for it (see p. 138). Another important factor is the chemical form of the element (Chapter X).

It is worth noting that ^{14}C[96] and ^{3}H[89,97], which are so important in biochemistry, are only 'slightly poisonous' since the energies of their radiations are small, and their biological half-lives are short. However, both the biological half-life and the preferred position of deposition in the organism must depend upon the chemical nature of the labeled material.

4. Safety Measures

These considerations may well be summarized by saying that the uptake of radioelements by the organism should be carefully avoided. Suitable precautionary measures have been described in the literature[67,98–102]. In the radiochemical laboratory one should not eat or drink, nor, if possible, smoke or use cosmetics. The hands should be thoroughly cleaned on leaving. Radioactive residues must be rendered harmless in suitable ways; in biochemical investigations with labeled atoms the activity is usually so low that the residues may be flushed down the sink. Glass vessels, etc., which have become radioactive, are cleaned as promptly as possible in appropriate ways—usually with acid or oxidizing agents—depending on the nature of the radioelement. Contamination of the tables is prevented by covering them with glass or with paper sheets; if they nevertheless become radioactive they are scrubbed with soap and water, or if necessary polished with wet abrasive. Such decontamination of the laboratory is of course also necessary to avoid erroneous results.

Utmost care must be exercised when dispensing labeled material to human beings for investigative purposes. This should be done only in exceptional cases, and only after the responsible biochemist has made certain by a careful consideration of all the factors involved that no injury is to be feared.

The same is true of the diagnostic and therapeutic use of radioelements. A discussion of such uses does not lie within the province of this book, but reference may be made to the numerous cases of cancer ascribable to the thoughtless and (even in the light of the state of knowledge prevailing at that time) irresponsible administration of radium[103–110] and thorium (as thorotrast)[107, 109, 111–114].

The destructive action of ionizing rays on living matter has been considered, studied, or in some cases employed practically, for purposes of sterilization and disinfection and for the preservation of food. In these connections we refer the reader to the literature[115].

5. Changes Produced by Radiation Effects

We now return to the question touched upon in Chapter I, namely, the extent to which the irradiation effects the properties of a particular biological system which is under investigation. In the literature quoted in section VII, *1* a large number of often contradictory observations have been collected. This is due to the great variability of living organisms and to the difficulty in obtaining reproducible preparations of substances of biological origin. Unfortunately some workers in this field have published too readily and without sufficiently considering the statistical significance of their results.

Two things are necessary to determine whether or not radiation effects are to be feared in the system being studied. In the first place, the approximate radiation dose must be known, and in the second place, it must be possible to estimate the sensitivity of the system.

The approximate radiation dose for an aqueous system exposed to β-rays (see below) can be calculated by a rule-of-thumb formula of the type

$$D = \frac{55AE}{3v}$$

where A is the activity of the substance in curies, v the volume of the system in ml, E the maximal energy of the radiation in electron volts, and D the dose rate (in r/day). The numerical factor 55 arises from the conversion of electron volts to roentgens and the choice of the day as the unit of time; the factor 1/3 is the approximate ratio of the average energy to the maximum energy of the β-rays. Since only a small fraction of the rays emerge from the system, unless its volume is very small, absorption of all of the energy is assumed.

As a numerical example, let us assume that an *in vitro* system contains 1 microcurie of radiophosphorus per ml. The energy of the β-rays from phosphorus is 1.7×10^6 eV. The formula then gives:

$$D = \frac{55 \times 10^{-6} \times 1.7 \times 10^6}{3} \simeq 30 \text{ r/day}$$

Dose rates of α-rays can be estimated in an analogous way. The factor 1/3 must be omitted, however, since the average energy is here equal to the maximal energy. Also, α-rays have more effect on living systems than β-rays (RBE $\gg$ 1; see p.60). In the calculation of γ-ray doses, one must take account of the fact that only a very small fraction of the radiation is absorbed within the system under investigation.

As far as the radiation sensitivity of the system is concerned, only semi-quantitative data are at present available. Whereas damage *in vivo* or in tissue culture can, in certain cases, be produced by only a few roentgens (see above), solutions and gels of organic materials, including solutions of nucleic acids and enzymes, are strongly affected only by larger doses; the damage decreases as the content and effectiveness of protective agents in the system increases. It is to be expected that organized systems, like the particulate fractions of cells, will have intermediate sensitivities. In cases of doubt, control experiments are needed; these may depend on the application of further (increasing) doses of radiation and examination of the changes produced, if any, in the system. External (γ-) radiation sources or internal sources (tritium) may be used.

A peculiar kind of 'radiation damage' is induced intentionally in certain experiments with DNA. When radiophosphorus incorporated into DNA (*e.g.* in bacteriophages[116–121] or bacterial cells[121–123]) decays with β-ray emission according to $^{32}\text{P} \rightarrow {}^{32}\text{S}$ (inactive), the DNA may or may not lose its biological activity. The results of such experiments lead to conclusions on how much damage the DNA molecule must suffer to lose its typical biological activity. This type of 'radiation damage' (suicide effect) may be distinguished from the ordinary damage caused by the general radiation field within the biological unit by varying the specific activity of the phosphate used to grow the radioactive DNA. 'Generalised damage' will not depend on the specific activity, but 'specific damage by nuclear transformation' will.

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CHAPTER VIII

THE MEASUREMENT OF RADIOACTIVITY

1. Fundamental Principles

The radiation emitted by radioactive substances is, in contrast to visible light waves or radiowaves, ionizing radiation; it is therefore able to remove electrons from atoms or molecules. This phenomenon has been used for the measurement of the radiation ever since the first work of the Curies: by various means the presence of the ions and free electrons, which the radiation produces in the sensitive part of the measuring apparatus, is determined. Even to this day most instruments are based on this general principle.

The simplest arrangement involves the measurement of the ionization which results from the radiation in a gas-filled container. By various methods, which will be discussed presently, it is possible to increase the number of ionized particles in the gas above the number produced by the primary interaction. The photographic methods, in which the radiation is determined by the blackening of a photographic plate, also depend upon the ionizing action of the rays, which makes the grains of silver bromide sensitive to the developer.

The scintillation counters, on the other hand, which have become very important in the past few years, do not make use of ionization, but rather of the excitation of molecules caused by their reaction with the radiation. In this case the electrons are not knocked out of the molecules, but raised to higher energy levels within them; they later return to their original level, with the emission of light. This light is observed, and the intensity of the radiation is determined from the number of the flashes of light.

There can be various reasons for the measurement of radioactive rays. In biochemistry the most frequent reason is the wish to determine the activity of a sample. Here one must distinguish between absolute activity and relative activity. The measurement of the absolute value is seldom attempted; the answers to biochemical questions can almost always be obtained by measuring the activity relative to that of a standard sample which emits the same sort of radiation. Relative measurements are naturally much simpler than absolute ones. The standard is often a known aliquot of the radioactive material which is introduced into the experimental system; for example, the amount of the radioelement found in a compound formed by the body, or present in an organ, is compared to the amount originally introduced into the body.

The number of 'counts per minute', obtained with a certain experimental arrangement, is often taken as a measure of the relative activity when 'counters' (especially Geiger counters) are used. The measuring arrangement must be maintained constant during each series. However, if this is not possible, as is the case when the thickness of the sample varies, corrections must be applied.

Absolute activities can be calculated from the measured activities if the fraction measured is known. A direct calculation of this 'yield' is difficult for the most frequently employed types of instrument; it depends on many factors, including, for example, the energy of the radiation, the shape of the instrument, and the position of the sample. Standard samples of known absolute activities are therefore mostly used for the determination of this yield.

In other cases, it is necessary to determine the type and energy of the radiation. Such measurements, which are required in biochemical work only when more than one radioelement is present, serve to identify the radioelement. It may be necessary, for example, in cases of double labeling, or in the examination of the metabolism of a mixture of fission products. A physical method of identification consists in the determination of the absorbability of the rays, *e.g.*, by measuring the intensity through absorbing foils (see also p. 39); chemical identification can be made by observing in which fraction the active element appears after analytical separation[1].

Finally, it may be necessary to measure the energy which the radiation imparts to a specific portion of a system (dose). For this purpose, the number of ions formed per unit time in a given volume of a suitable gas can be measured, and then the corresponding value for the experimental system can be calculated, taking account of the density and chemical composition of the two media. Experience has shown that the number of ions formed is, as a first approximation, generally proportional to the energy absorbed; in other words, that the relationship between ionization energy and excitation energy is only slightly dependent on the nature and energy of the radiation or on the composition of the medium. Measurements of the doses can be used to decide whether changes can be produced within a tissue by radioactive rays (p. 4); they also form the basis for protection against radiation (p. 58). This type of measurement cannot be discussed in detail here however[1–5].

In this review, it is only possible to present the fundamental principles of measurement. The object is to provide the biochemist with a general orientation and to aid him in choosing his methods of measurement. For details of the instruments and the procedures for using them, we must refer the reader to the surveys, which will be cited in the following sections.

2. Ionization Chambers

Ionization chambers are not used in biochemistry on a large scale. Yet a short description of their mode of action is advisable because it will further the understanding of the proportional counter and of the Geiger counter, the most important measuring device in radiobiochemistry. The reviews usually apply to all three types of apparatus[6–11].

Ionization chambers operate on the principle, mentioned previously, of the measurement of the number of ions and electrons produced by the radiation in a gas-filled space. This is done by applying an electrical potential across the chamber by means of two electrodes, which, in the simplest case, consist of two parallel metal plates. The electrons, or the negative ions formed by the addition of electrons to atoms or molecules, then move toward the positive electrode (anode), and the positive ions toward the negative electrode (cathode). The motion of the charged particles constitutes a current, which can be measured directly with a galvanometer, or amplified electronically. Instead of measuring the intensity of the current, it is also possible to charge the chamber to a given potential, and then to measure the speed of the discharge with an electroscope.

The formation of ions in the gas chamber is accompanied by a spontaneous discharge of the ions, since the negative and positive particles attract one another and 'recombine' with the formation of neutral atoms or molecules. Therefore the ions are collected on the electrodes better when the electric field is strong, because the faster the particles travel the less opportunity they have to meet and to recombine. Above a certain minimum value of the field strength (potential gradient) essentially no recombination takes place. The current through the chamber is then determined solely by the number of ions formed per unit of time in the sensitive volume of the gas; this 'saturation current' constitutes, therefore, an unambiguous measure of the intensity of the radiation, and hence of the activity, if the nature of the radioelement and the experimental geometry remain fixed.

Thus there occurs in this region of saturation a long plateau in the value of the current, when it is measured as a function of the voltage (Fig. 5,

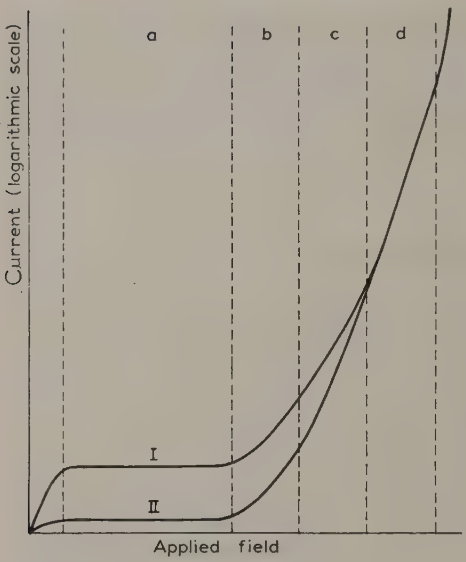


Fig. 5. Schematic curve of current vs. voltage in ionized gases[13].

I. Curve for α-particles of given energy.
II. Curve for β-particles of given energy.
a) Saturation current region, b) proportional region, d) Geiger region.

portion a of the curve). We shall soon discuss the reasons why the plateau has an upper limit. In practice, the ionization chamber is always used in the region of the plateau—never below it—because in this case maximal current intensity is obtained, and also because the current is then independent of variations in the voltage. The current can be measured, *e.g.*, by means of the vibrating reed electrometer[12]; this technique has been used in biochemistry.

When weak sources of radiation are used, it is possible, instead of measuring the 'integrated' current, to count the individual pulses which result from the absorption of individual ionizing rays. This can de done if each individual pulse exceeds a certain minimum size. In practice this usually happens with α-particles, since, because of their short range, they readily give up all of their quite appreciable energy inside the chamber; in other words, their specific ionization (number of ions formed per unit length of track) is high.

β- or γ-rays have a low specific ionization, forming fewer ion pairs per centimeter of path length than do α-particles. Therefore, counting chambers are not suitable at all here, and 'integrating' chambers can be used only when the intensity of the radiation is considerable.

The samples may be in the form of solid layers. Another important use of ionization chambers is for the measurement of the gaseous emanations (radon, thoron, actinon) which can be introduced as such into the chamber; all of these emanations are α-emitters. The determination of other radioactive inert gases (krypton, xenon) and of gaseous compounds of radiocarbon and radiohydrogen with ionization chambers has also been described; these substances are β-emitters.

3. Proportional Counters

If the field applied to the gas chamber is further increased, the end of the plateau, *i.e.*, of the saturation current region, is finally reached. Thereafter, a rapid increase in the current is observed (portion b of the curve in Fig. 5). This increase is due to a new physical effect, ionization by collision (gas amplification). Only the fate of the electrons set free by ionization will be discussed here.

The electrons which are attracted to the electrodes collide many times with neutral atoms or molecules during their passage through the gas. They thereby lose a part or all of the kinetic energy which they had acquired by the action of the field. After each collision they are, however, accelerated again, only to lose the newly-gained energy in another collision, and so on, until they finally reach the electrode. When the field strength is large enough, the electrons acquire so much energy between collisions that they ionize atoms or molecules which they strike.

In this way, a primary electron gives rise to a number of secondary electrons which are also accelerated, and which also collide with other particles and ionize them. The result is an avalanche of electrons, the magnitude of which depends upon the strength of the field. The factor by which the electrons are multiplied is indicated by the size of the discharge. This size is determined by the number of electrons which reach the anode as a result of the absorption in the gas of a radioactive ray of a given kind and energy.

In the region b (Fig. 5) the magnitude of the pulse (discharge) does indeed increase with the strength of the field, but at any given value of the potential it remains proportional to the number of primary electrons produced by the radiation. This is easy to understand: the more primary electrons are present, the more secondary electrons are produced, other factors remaining constant. So-called ‘proportional counters’ operate in this region.

Proportional counters, unlike ionization chambers, are usually constructed so that the potential is applied between a cylindrical metal casing and a thin metal wire along the center of the cylinder. The wire is positively charged. Since the current through the gas is increased by the formation of secondary electrons, it is possible to measure individual β- and γ-rays with this instrument, while this is impossible with an ionization chamber. When individual α-rays are counted, the amplifying circuits do not need to be nearly as powerful as in the case of ionization chambers. Discrimination between rays of different energies is practicable with the proportional counter as well as with the ionization chamber, and can of course be extended to β-rays and γ-rays.

The proportional counter is nevertheless relatively seldom used in the biochemical laboratory. It is much simpler to use the Geiger counter, which is sturdier and more reliable. Some authors have, however, determined radiocarbon with proportional counters filled with CO_2 (p. 91). Flow-counters (p. 80) are also often operated in the proportional region.

4. Fundamentals of the Geiger Counter

When the voltage is still further increased, the proportional region is left, and the sizes of the pulses are no longer proportional to the numbers of primary electrons. The discharge produced by each ray does indeed increase as the voltage rises, but the relative increase is the smaller, the greater the primary ionization (portion c of the curve in Fig. 5). The pulses arising from primary events of different magnitudes approach one another. Finally the region d of the curve is reached, where the size of the pulse is independent of the amount of primary ionization, *i.e.*, all discharges are nearly equal in magnitude. This is the ‘Geiger region’, in which the Geiger counter operates.

The basic advantage of the Geiger counter is that the amplification inside the gas chamber is so very great that the external (electronic) amplifier may be very simple. It is simpler, and hence more stable, than the proportional counter. In practice, each primary ion pair can yield 100 million secondary ion pairs in a Geiger counter.

The disadvantages of the Geiger counter—which are, however, not very important in practice—include, firstly, the necessity of having high voltages. In the second place, since all pulses are alike, no distinction can be made between pulses due to different physical processes. In particular, one cannot distinguish between the effects of different types of rays, or even between the ‘true’ counts caused by the sample, and the ‘false’ counts, which are due to radioactive impurities in the sample and the environment, or to cosmic rays.

5. The Background

In order to take account of ‘false’ activity, it is necessary to measure the so-called ‘background’ and to subtract it from the measured activity. This is done by observing the pulses under the same conditions as will occur in the test, but with the sample omitted. (Backgrounds exist in the cases of ionization chambers and proportional counters as well. However, because of the possibility of distinguishing between counts due to different rays they are less important.) The background can be decreased by shielding the counter tube with iron, or preferably, with lead. The background of a typical well-constructed Geiger counter for biochemical work, with several centimeters of lead shielding, may amount to 5–50 counts per minute (c.p.m.), depending on its size; the background of an unshielded counter is approximately twice as great.

The effect of radiation from the environment is largely suppressed by shielding. The complete elimination of the effect of cosmic radiation cannot be achieved by shielding, however, since the hard component of this radiation is extremely penetrating. (One experiment—using a photographic plate as detector (see below)—was performed in a mine 600 m below the earth's surface in order to reduce the background[14]). If the background is to be reduced radically—say, to one-tenth of its original value—, both shielding and an anti-coincidence circuit must be used; this device was first employed in physical investigations[15], later for radiocarbon dating[16], and finally for biochemical purposes[17].

In this arrangement, the counter tube is surrounded by a ring of auxiliary counters. When a penetrating cosmic ray, or a shower of rays, triggers the measuring counter, almost always at least one of the auxiliary counters is affected as well. The counter tubes are so wired ('anti-coincidence') that only those discharges are counted which appear in the measuring counter exclusively, and are not accompanied by simultaneous discharges in any of the auxiliary tubes. The general problems of low-level counting—not only with Geiger counters—have been reviewed[18, 19].

6. The Plateau

From the standpoint of reliability and reproducibility of the measurements with a Geiger or proportional counter, the observed values must be largely independent of the potential applied. Otherwise the results would be affected by unavoidable fluctuations of the voltage. Actually, it is to be expected that the number of counts will, within limits, be independent of the voltage, since the latter should affect only the magnitude of the individual discharges.

To illustrate this point, Fig. 6 gives the number of discharges per unit time, caused by radiation of constant intensity entering a Geiger counter, as a function of the voltage. (Fig. 5 gave the size of the individual discharges as a function of the potential.) Fig. 6 thus represents a typical 'characteristic curve' of a counter tube.

Good Geiger tubes have a long voltage range (as is shown in the figure),

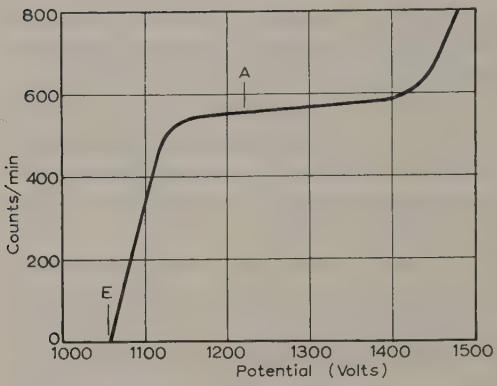


Fig. 6. Typical characteristic curve of a Geiger counter.
(E: threshold, A: suitable working-point).

in which the number of counts is essentially independent of the voltage, *i.e.*, a 'plateau'. (This is also true of ionization chambers and proportional counters). The slope of the curve in this region should in practice never exceed a few percent per 100 volts, and can closely approach zero (less than 1/2% per 100 V). The plateau should extend over a range of several hundred volts. For measurement, the voltage is usually chosen to be about one-third of the distance from the beginning of the plateau ('working point').

For each tube the shape of the plateau is checked from time to time. A deterioration gradually occurs with most counters. This deterioration is especially marked when air leaks into the tube because of faulty seals, but in addition the electrical discharges decompose the organic substances present as quenching agents in the gas filling (see below).

If it is suspected that the sensitivity of a counter at its working point has changed with time, it should be checked with a standard sample. The standard sample may contain, for example, some uranium; the β-radiation of the daughter substance of uranium (uranium X) is counted. The values obtained with the counter can then be corrected on the basis of the measured activity of the standard sample.

The threshold voltage increases with the pressure of the gas in the counter tube. On the other hand, no satisfactory plateau is obtained at all if the pressure is too low. Suitable gases for use in the tubes are, for example, the inert gases, and, to a certain extent, hydrogen. Oxygen, even in small amounts, is extremely deleterious. The factors affecting the suitability of the gases are discussed in the literature on counter tubes.

7. Quenching of Geiger Counters

Every Geiger counter has a tendency to give a continuous discharge instead of the desired, intermittent discharges brought about by individual rays. In view of the large gas amplification, this is hardly surprising. Electrons which escape neutralization give rise to fresh avalanches; in particular, the ultraviolet light, which is generated in the counter tube, liberates additional electrons from the metal walls by virtue of the photoelectric effect. In order to avoid disturbances of this nature, and to restore the tube to its normal condition as quickly as possible after the discharge (*i.e.* to limit the 'dead time'), it is necessary to quench the discharge automatically. This can be done either externally (electronically), or internally by adding a quenching gas to the filling of the tube. Faulty quenching is shown by an excessive slope in the characteristic curve, *i.e.*, by the absence of a true plateau.

External (electronic) quenching is accomplished by sharply decreasing the applied voltage automatically as soon as discharge begins. This prevents further multiplication of ions, and the free electrons already present disappear. The tube then 'recovers'. Various types of quenching circuits have been developed, *e.g.*, the Neher-Harper and the Neher-Pickering circuits.

Internal quenching (self-quenching) is brought about by addition of a gas which absorbs ultraviolet light without forming ions and electrons. Alcohol vapor and halogens are suitable for this purpose. A typical self-quenching filling consists of argon at 80 mm and alcohol at 15 mm Hg; considerably higher pressures are often employed now. Whereas organic quenchers are gradually decomposed by the discharge, and tubes containing them must be refilled after a certain length of time (10^8–10^9 discharges), or the tubes discarded if they can not be refilled, the halogens remain unaffected.

The dead time extends from the beginning of a discharge to the end of the quenching; during this period the tube is not sensitive to further radiation. The existence of such a dead time leads to losses in the measurement of high intensities, where the rays follow one another in rapid succession. With an argon–alcohol filling, the dead time may be 1/5 millisecond for example. Since the pulses, because of the statistical nature of the decay, sometimes occur at shorter intervals than the average, the limitation in the resolution due to the dead time becomes apparent even below 5000 discharges per second in the given case.

In the early days, the measurable intensity was limited even more strictly by the inertia of the mechanical counting device which ultimately records the number of discharges. However, electronic 'scalers' are now generally used. The scaler causes the mechanical counter to be triggered by a known fraction of the pulses only, for example, by every sixteenth ($2^4 = 16$) or, (in the case of 'decadic' scalers) by every tenth, hundredth, etc. pulse. The number appearing on the mechanical counter must then be multiplied by the scaling ratio. The number of individual pulses of the tube in excess of a multiple of the scaling ratio can be determined electronically.

Losses caused by excessive intensity can, to a certain extent, be estimated by empirical corrections. These corrections are determined by increasing (or decreasing) the intensity by a known factor, and noting the increase (or decrease) in the counts. The intensity can be increased, for example, by superimposing radiation sources of known activities; alternatively, an originally very active source with a known half-life can be allowed to decay. There is no certainty, however, that correction curves will remain unchanged over long periods of time. It is better to decrease the intensity of the radiation from the sample by diluting solutions, interposing absorbers, decreasing the effective solid angle, etc.

8. Design of Geiger Counters—External Counters

As has been noted above, Geiger counters are sensitive to all kinds of ionizing radiation. Their construction is, however, tailored to the radiation to be measured. Since α-rays have a low penetrating power, the tubes used to measure them (and also very soft β-rays) are equipped with thin windows (usually of mica), through which the rays can enter the sensitive volume. Alternatively, the sample can be placed inside the tube, so that no absorption by the walls or a window can occur. In the latter case it is necessary to pump out the tube and refill it with gas after every measurement. We shall return to this type of counter in the next section.

The measurement of γ-rays, on the other hand, is rendered difficult by the low absorption of the radiation. In order to be counted, the γ-rays, like all others, must cause ionization, *i.e.*, produce free electrons by some means or other. However, since the γ-rays interact with matter only slightly, and are very penetrating, the probability that an electron will be set free from the wall or the gas in the counter by a γ-ray is very small. It is of the order of magnitude of 1%, and can be increased only slightly by special design of the tube[19a]. γ-rays can be measured far more accurately with a scintillation counter (p. 81).

The thickness of an absorbing layer, like that of the mica window of a counter, is often expressed as weight per unit area (g/cm^2) so that different materials can be easily compared. The thickness of the sample, which determines the self-absorption, as will be discussed below, may advantageously be expressed in the same units.

It is easiest to measure β-rays which are not too soft. The radiation from most β-emitters, including radiosodium and radiophosphorus, penetrates the mica window and even the counter wall fairly easily. For example, a rather thick mica window of 10 mg/cm² (=1/30 mm) absorbs only 8% of the β-particles from ^{24}Na. The same window absorbs as much as 92% of the much softer β-rays of long-lived carbon. The β-particles from tritium, which are particularly soft, cannot be counted at all with external counters.

The α- and β-particles which penetrate into the sensitive region of a counter are—in contrast to γ-rays—almost 100% effective in producing at least one pair of ions, and thereby causing a discharge.

The solid angle through which radiation can enter an external counter is greatest in a measuring arrangement (Fig. 7) where the sample is almost in contact with the metal wall; it may then be as high as 30%. For window counters (because of their shape also called 'bell counters') a 'geometric yield' of only 5–10% may be expected (Fig. 8). Yet in practice window counters are preferred, because they are more stable, the positioning of the samples relative to the counter is more reproducible, and it is easier to interpose absorbing sheets. The samples to be counted are usually in the form of solid layers. The preparation of the sample will be discussed on p. 88.

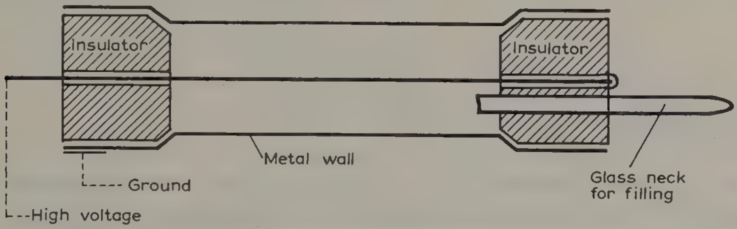


Fig. 7. Longitudinal section of a wall counter.

One interesting type of external counter is the so-called 'liquid counter' [20–23], which is a kind of wall counter, and is therefore in no way analogous to the gas counters (see below). The activity of the liquid is measured by immersing the counter wall, which in this case usually consists of a glass tube surrounding the cathode, in the liquid. There are also double walled counters, where the liquid is placed in the annular space.

The great advantage of the liquid counters lies in the ease of preparing and measuring the sample. Excellent reproducibility of the measurements is insured by the fact that the liquid automatically assumes the same spatial position relative to the counter every time. The disadvantage lies in the relatively low sensitivity of the measurement when dissolved radio-

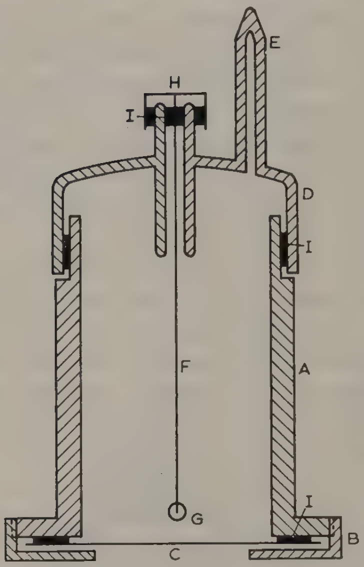


Fig. 8. Cross section of a window counter[13]. A. Brass casing, B. gasket, C. mica window, D. glass cover, E. sealed-off neck for filling, F. tungsten wire, G. plastic bead, H. metal closure cap, I. plastic seals.

elements are being measured, as is usually the case. The absorption of the radiation by the solvent is appreciable, and furthermore the average distance of the radioactive atoms from the wall of the counter is greater than it would be in absence of a solvent.

9. Internal Counters

Internal counters, into which the active samples are introduced, possess the fundamental advantage that absorption in the wall or window of the tube is eliminated. They also enjoy the advantage of a greater solid angle over which the rays are counted. On the other hand, the tube must of course be refilled after the introduction of each sample. The refilling takes time and effort; it is also necessary (except in the case of flow counters; see below) to determine the plateau again after each filling, in order to be sure that the apparatus is working properly, and to adjust the voltage accordingly.

For biochemical purposes the flow counter is the most important kind of internal counter for solid samples[24, 25]. In order to make the refilling of the counter as simple as possible, the tube is not sealed, and a special gas is allowed to flow through the tube slowly under a slight excess pressure. *E.g.*, a mixture of helium and isobutylene can be employed. The relatively high pressure naturally makes it mandatory to employ a high voltage; for this reason the flow counter is often operated in the proportional region rather than in the Geiger region. Ordinary methane can in this case be used to fill the counter. In work with flow counters, attention should be paid to static charges[26, 27].

Although there is no absorption by a window in a flow counter, the self-absorption in the sample remains; in the case of radiocarbon, for example, this is generally the most important reason for the small counting yield. Considerably higher yields are obtained with 'gas counters', filled with radioactive carbon dioxide (p. 92) or with tritium compounds (p. 94), and operated in the proportional or Geiger regions[28]. Here self-absorption does not occur; moreover, the useful solid angle is almost 100%. This type of instrument is therefore particularly suitable for sensitive routine determinations in biochemistry. However, measurements with gas counters require more time and effort than those with flow counters, or, especially, those with window counters.

10. Scintillation Counters

A primitive form of the scintillation counter played an important role in the early days of the study of radioactivity. Later, the scintillation counters became unimportant for a long time, and were superseded by ionization chambers, proportional counters, and Geiger counters. In the last decade, however, the scintillation counter has been revived with new (electronic) auxiliary equipment, and has enjoyed some notable triumphs. The chief importance of the scintillation counter in biochemistry lies in the fact that it is the only counter which permits the determination, with good yields, of γ-radiation [8, 9, 29–31].

All radioactive radiations, when absorbed by suitable materials ('phosphors'), excite them to a short-lived luminescence[32]. In its original form, the scintillation counter was used for counting only α-particles, which gave rise to flashes of light distinctly visible to the naked eye. At present, the flashes are detected and counted with the help of a photocell. Even the penetrating γ-rays can be registered by using a large phosphor. This must be transparent so that the light is not absorbed within it.

The phosphors may consist of inorganic salts (*e.g.*, large crystals of sodium iodide activated with thallium) or of aromatic compounds (*e.g.*, anthracene). Some organic phosphors may be used as solutions in liquids or in plastics[33]. A good phosphor is characterized by high yield of light and rapidity of light emission (absence of afterglow).

The electrons released in the photocell are amplified by passage through an electron multiplier attached to the cell (Fig. 9). This yields a large impulse for each incident electron, and the impulses are counted[34].

The rays usually penetrate the phosphor from without; the sample may

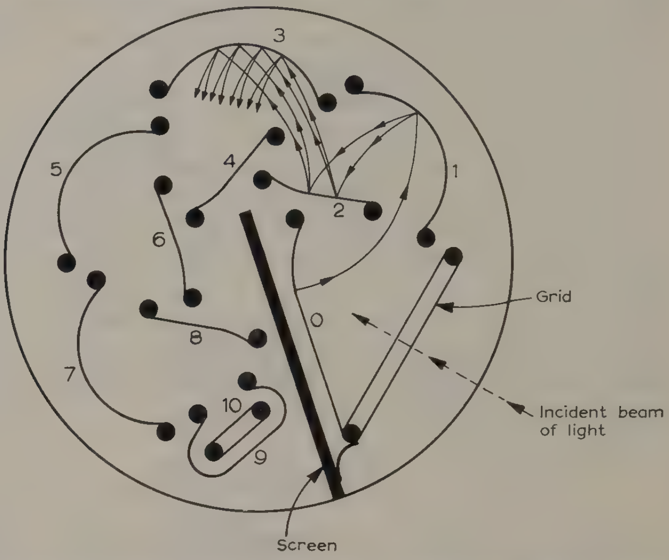


Fig. 9. Diagram of the RCA photomultiplier. The cathode is numbered 0, and the anode, 10. The 'dynodes' 1–9 are intermediate electrodes. The screen and grid afford electrostatic shielding.

be solid, liquid (p. 21) or gaseous[35]. From the standpoint of yield, it is an advantage to sink the sample into the depression of a well-type phosphor (Fig. 10)[36, 37].

If the radiation is soft, and is to be counted with good yield, the active material may be dissolved in a liquid phosphor[38, 39]. This procedure will be discussed in the sections on the measurement of carbon (p. 91) and hydrogen (p. 94).

A disadvantage of scintillation counters is their high background, mainly due to the spontaneous emission of electrons in the multiplier[40]. The background can be decreased by special measures, including the cooling of the multiplier and the use of so-called coincidence circuits.

In the latter case, instead of a single photocell a pair of cells, with attached multipliers, are employed. The circuit is such that a count is registered only when both photocells give discharges at the same instant. This only happens when a true flash is given by the phosphor; the flash is 'seen' by both photocells at the same moment. On the other hand, when an impulse arises in one of the multipliers as a consequence of the spontaneous emission of an electron, it is unlikely that the same thing will happen in the other multiplier at the same time. Only a single impulse arises. This does not affect the coincidence circuit, and is not recorded.

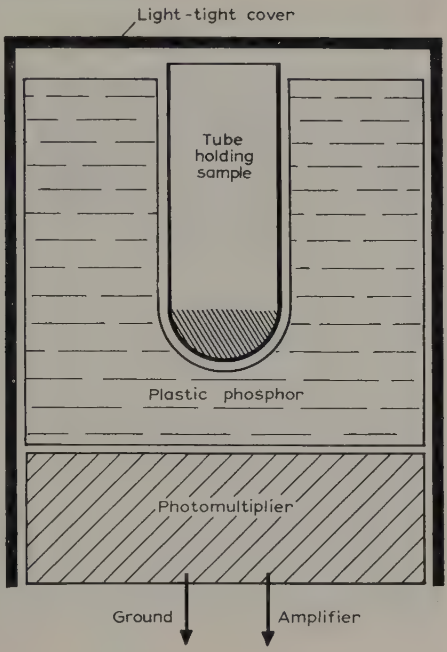


Fig. 10. Diagram of a well-shaped scintillation counter. The radioactive material is in the shaded area.

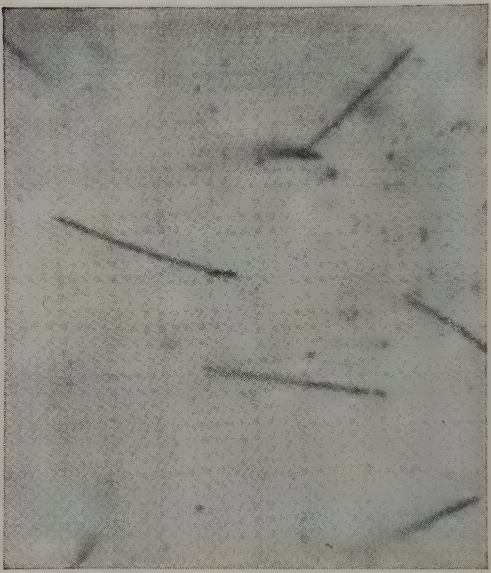
A special application of the scintillation counter is to the determination of the content of γ-emitters in living organisms of large size, for example, in man. The object being tested is brought into contact with large phosphors, or even surrounded with several hundred liters of a liquid phosphor [41–46] (*cf.*, p. 127). The use of suitable screens permits the determination of the spatial distribution of the γ-emitter, *e.g.*, iodine, sodium or potassium, in the body[47] (see Fig. 12b). The measurement of the radioactivity of the human body with scintillators is an important aid in the assessment of internal contamination by radioelements[48].

Two radioelements can be determined in the presence of one another if the energy of the rays, and hence the size of the flashes of light, are sufficiently different[49]. A discriminator is required for this purpose. The light flashes may be produced by β- or γ-rays. In this way, the pairs ^{51}Cr and ^{59}Fe, ^{24}Na and ^{42}K, or ^{51}Cr and ^{131}I have been determined (see also p. 127)[50, 51].

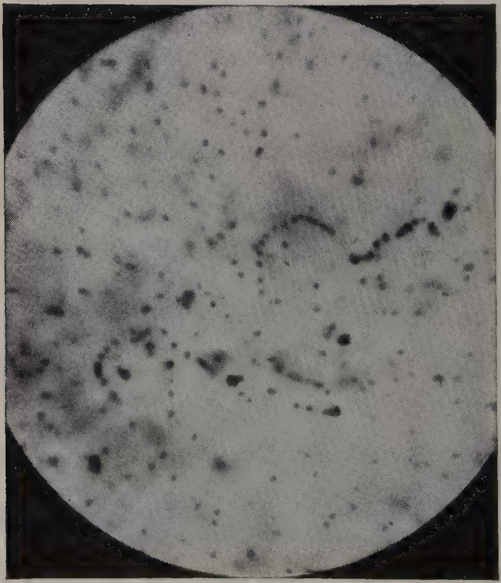
11. Photographic Methods

As the last group of methods of measurement, the photographic procedures are to be mentioned[49, 52–56]. To photography we owe the discovery of radioactivity. In the last decade, since the production of 'concentrated'

Fig. 11. Tracks of individual rays in a nuclear emulsion (Photograph by K. Jenkner and E. Broda, enlarged approx. 1300 ×).



a) α-particles from uranium. (Most of the tracks run at an angle to the plane of the picture, and are therefore out of focus over part of the path).



b) β-particles from rubidium.

emulsions, photographic methods have again come very much to the fore. These emulsions, which are produced for work with radioactive materials, contain 80% or more of silver bromide, so that little of the radiation energy is lost by absorption in the gelatin, and the grains of silver are in close proximity. Such plates record the tracks of single α- or β-particles extremely clearly (Fig. 11a and b[57]).

The photographic measurement of radioactivity can be carried out either on solutions, which are by nature homogeneous, or on substances which possess a definite structure.

In the first case, the determination of the activity serves to determine the concentration of the radioelement in the solution. The solution is brought into contact with the emulsion in some suitable way, the plate is dried, and after a given time (hours or days) it is developed and fixed. The blackening can be determined with a densitometer, or the individual tracks can be counted. In the latter case, the shape and length of the tracks permits radiations of different kinds and energies to be distinguished. Counting of the tracks in an emulsion is naturally more tedious and less accurate than counting impulses with a counter, but the sensitivity of the method is very high: the tracks may be allowed to accumulate in the emulsion over a long period of time.

In order to let the dissolved material act on the emulsion one can, for example, dry a drop of the solution on the emulsion. It is also possible to soak the emulsion in the solution for several minutes, so that the gelatin becomes saturated, and to dry it. The first procedure has the advantage that the volume of the solution, in which the active material was originally present, is known; but the distribution within the emulsion is not uniform, so that the entire volume must be scanned. In the immersion procedure, on the other hand, because of the swelling of the gelatin and because of adsorption effects, the volume of the solution taken up is not known with any precision; the distribution within the emulsion is uniform, however. Both methods are applicable to α-particles but not to β-particles, because the latter give suitable tracks only in highly sensitive emulsions, and the sensitivity of the emulsion is decreased by treatment with solutions.

β-rays can be counted, however, by a 'capillary' method, which has been developed for carbon 14[58]. The active solution is sucked into a thin-walled glass capillary, over which an electron-sensitive liquid emulsion is poured. The emulsion is left to solidify. Now the β-rays, which penetrate the glass, give tracks, which are counted after developing and fixing the emulsion. The fraction of β-particles detected is determined by calibration, but is always quite high. The sensitivity of the detection of radiocarbon by this procedure is excellent, because the background is very

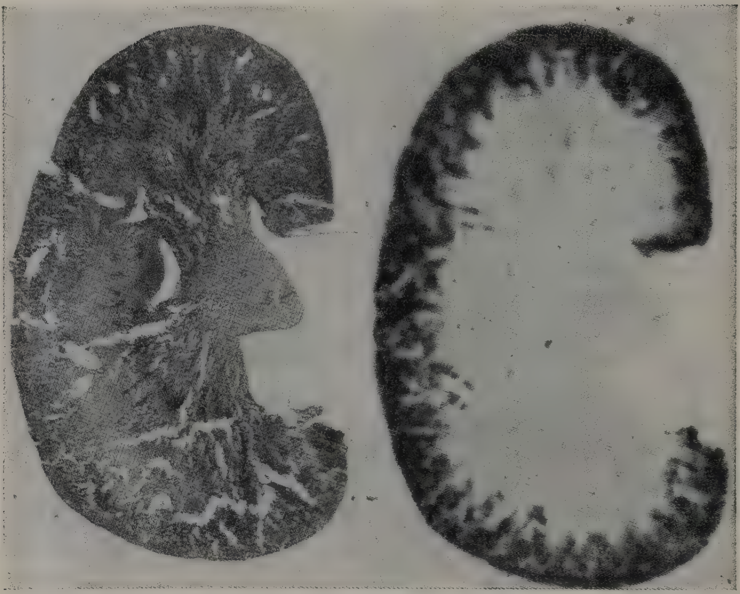


Fig. 12a. The localization of polonium in kidney tissue (from H. Yagoda, Measurements with Nuclear Emulsions).
Left: photomicrograph of a stained section.
Right: autoradiograph (α-rays).

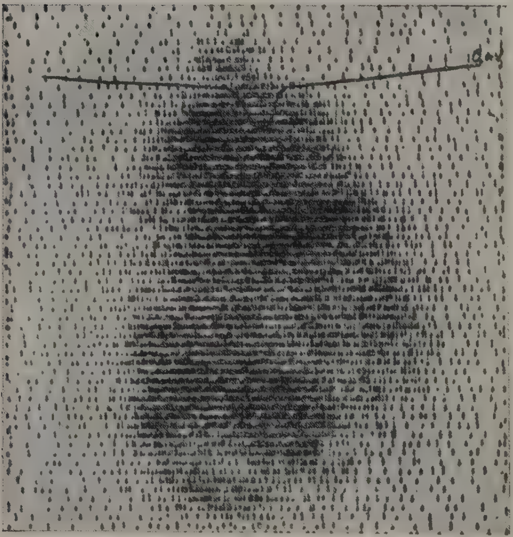


Fig. 12b. Scintigram of a patient with intrathoracic struma, who had been given radioiodine. The line represents the position of the collar bone (Photograph by H. Vetter).

small, 'false tracks' appearing seldom. Only a small quantity of solution can be employed, however. The procedure is therefore particularly suited to the determination of very small amounts of radiocarbon in small samples, *e.g.*, in chemical fractions from small organs of animals or plants.

For the determination of very small concentrations of plutonium in urine, this element is electrodeposited on a foil, a nuclear plate exposed to the foil for several days, and the number of α-ray tracks counted with an automatic scanner[59]. This procedure may well be applied to other radioelements.

The distribution of activity in a solid body with a definite structure is determined by autoradiography. A smooth surface of the sample is pressed against the photosensitive surface; it is advisable to interpose a thin foil to prevent chemical interaction. The distribution of the radioactive substance in the sample is recognised from the blackening (see Fig. 12a). Autoradiography was introduced into biology by Lacassagne[60] and has since assumed tremendous importance[11, 52, 53, 54, 61–63]. Even the intracellular distribution of radioelements, for instance the uptake of radioactive thymidine by chromosomes[64] (p. 262), has been followed. Not only α- and soft β-emitters (H, C and S)[64–67], but also hard β-emitters (Co and P)[14, 68] have been applied. Radioelements in chromatograms are located by autoradiography[69] (pp. 23, 169).

Especially suitable for the purpose of autoradiography are 'stripping films' which are distinguished by their thin support, so that the scattering of β-rays in it is unimportant and resolution is very good. The emulsion is placed upon a thin backing of gelatin or cellulose acetate[54, 70, 71].

Under some circumstances autoradiography can be extended to non-radioactive substances. The distribution of boron in tissues can be determined by exposing the tissue slice with the emulsion to slow neutrons. The fast ions, which are produced during the absorption of neutrons by boron—reaction ${}^{10}_{5}B$ (n, α) ${}^{7}_{3}Li$—blacken the emulsion[72, 73].

Many attempts have been made recently to put autoradiography on a quantitative basis. It is possible, though tedious, to count the blackened grains of silver[74]. Automatic scanning of the plate with a beam of light has also been proposed[75]. Another procedure[65] consists in photographically integrating at all values of the abscissa, with the help of a cylindrical lens, the blackening of strips running parallel to the ordinate; the resulting secondary photograph (one-dimensional profile), arising from the series of parallel strips, is examined photometrically. Absolute measurements are possible if the blackening of the plate is compared with that produced by a standard sample containing a known amount of the radioelement[76, 77].

Instead of counting the grains or measuring the diffuse blackening, the tracks originating in a given region can be counted. This is easily done in the case of α-particle tracks[78, 79] but β-particle tracks have also been counted[80–82]. Radiocarbon and radiosulfur in yeast and algae[83], carbon in bone marrow cells[84] and phosphorus in particles of virus and of nucleic acid[85] have been measured in this way. The radioactive substance can be located particularly well if the autoradiography is followed by electron-microscopy of the same sample[86]. A distinction between different radioelements is possible on the basis of a distribution curve of the lengths of the α-particle tracks. This is true even when the radioelements are present in fairly thick layers of tissue; an example is the distinction among various decay products of thorium, arising from the deposition of thorotrast[87] (p. 63).

It has been proposed to interpose a thin, fluorescent screen between the sample and the emulsion. Thus the emulsion is blackened not directly by the radioactive radiation, but rather by the light given off by the screen. Since the absorption of each radioactive ray causes the emission of many quanta of light, increased blackening results[88–90]. In certain cases, it is an advantage to soak a radiochromatogram with a liquid scintillator before autoradiography (p. 81).

12. The Preparation of Solid Samples for Counting

The problem of the preparation of samples suitable for counting, especially by Geiger counters, has already been touched upon in the discussion of liquid counters and gas counters[1, 91]. When the latter types of counters are not used, it is necessary to put the active material in a reproducible manner into the form of a solid layer or (less frequently) a liquid layer. This sample is placed beneath the window or close to the outer case of the counter tube or into the tube. Care must be exercised to reproduce the geometry, *i.e.*, the relative position of the sample and the counter.

Since the self-absorption depends upon the thickness of the sample, quite wrong conclusions about the relative contents of radioelements would be obtained with samples of different thicknesses, especially in the case of soft, easily absorbed β-rays. (Because of the variation in self-absorption, great care must also be taken to use only dry, non-hygroscopic samples.) Moreover, part of the β-rays which travel toward the bottom of the sample are scattered back by the support and thus do enter the counter. The back-scattering is superimposed on the self-absorption. Various measures, depending on the circumstances, may be adopted to minimize errors due to the variation in self-absorption and back-scattering from sample to sample. These will be discussed primarily with reference to the most important case, that of the β-rays.

The solid sample is most easily prepared by evaporating to dryness a weighed or measured drop of a solution of the active material placed on a suitable support, such as a glass or platinum planchette (small plate). Care must be taken that the drop is always in the same position relative to the counter. If the concentration of the solution is low, self-absorption is unimportant. This simple procedure (*first* method) is, unfortunately, restricted to materials with high specific activities, because only then does a very thin layer emit sufficient radiation.

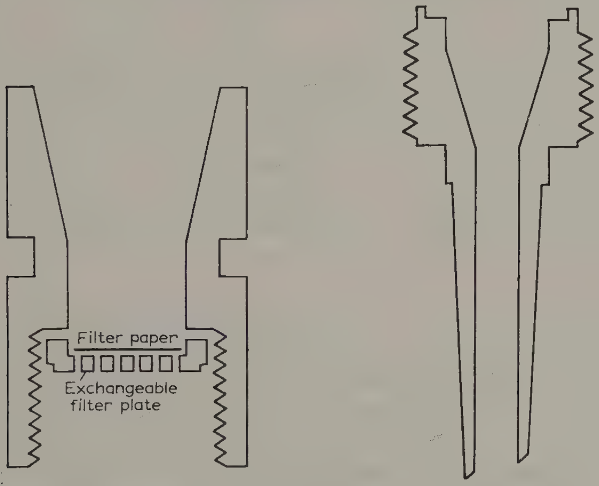


Fig. 13. Special filtration apparatus for radioactive precipitates[13].

More frequently is it necessary to precipitate the radioactive substance from a fairly large volume of solution; phosphate ion, for example, with magnesium and ammonia as $MgNH_4PO_4$. The precipitate is collected by filtration; it is advisable to employ a special device (Fig. 13) where the filter paper is placed on a removable metal filter plate. The precipitate, together with the paper and the filter plate, is placed in a suitable holder in a reproducible position underneath the counter. The precipitate may be centrifuged instead of filtered; centrifuge tubes with replaceable bottoms are useful. With these procedures layers are obtained, the thickness of which usually may not be neglected.

In the limiting case, the thickness of the layer exceeds the range of the β-rays ('infinite thickness'). The lower part of the sample then makes no contribution to the measured activity; in other words, the observed activity is not dependent on the thickness of the layer at all. The measuring yield is small, to be sure, but the measured intensity is always greater than that given by the method of 'thin layers'. This is the *second* method. When there is not enough material to prepare a layer of 'infinite thickness', one of the following methods must be employed.

In the *third* method, the thickness of the layer is always kept exactly the same; this is mostly too tedious for actual practice. In the *fourth* method, layers of different thicknesses are indeed employed, but the intensities are recalculated to standard values by means of a correction curve. The correction curve (Fig. 14, for example) varies with the energy of the rays and with the experimental procedure.

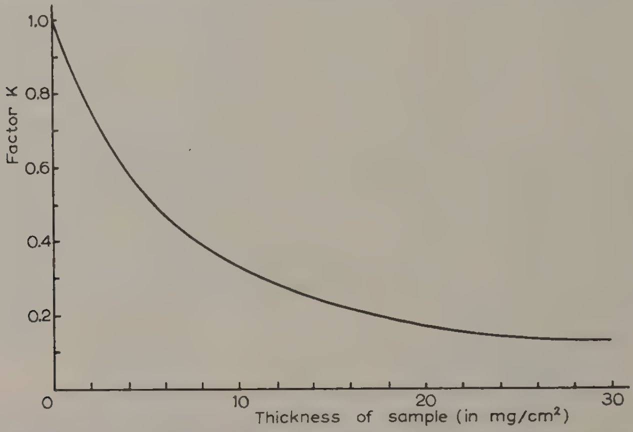


Fig. 14. Typical curve for the correction for self-absorption[13] (Radioelement, ^{14}C. Precipitate, barium carbonate. *K*, factor by which the measured activity must be divided in order to give the activity which would be measured if the same amount of radioelement were present in an infinitely thin layer).

The self-absorption of β-rays can be roughly estimated by an empirical equation of the form

$$I = I_0 \, e^{-\eta d}$$

where the self-absorption coefficients η for the radioelements ^{14}C, ^{32}P, ^{56}Mn have the approximate values 120, 3.0, 1.4 cm²/g. (The maximal energies of the β-rays are 0.155, 1.71, and 2.81 MeV.) I *and* I_0 are the intensities of the radiation with and without self-absorption, when the layer has a thickness of d g/cm². The self-absorption of other radioelements can be estimated from the energy of the β-rays. For a more detailed discussion, we refer to the appropriate literature surveys[1, 49, 92] (see also p. 79). The formula indicates, for example, that about 5% of the initial intensity is lost by self-absorption in a layer of 10 mg/cm² in the case of phosphorus, and in a layer of only 0.3 mg/cm² in the case of long-lived carbon.

With regard to the self-absorption of γ-rays, it may be mentioned here that only 5% of the radiation from a layer of Co, 200 mg/cm^2 in thickness, is absorbed; it is therefore often permissible to neglect the self-absorption of γ-rays. The self-absorption of α-particles, on the other hand, is extremely great, so that in their case only very thin or 'infinitely thick' samples can be used (methods *1* and *2*, above). More detailed descriptions may again be found in the literature[1, 92].

13. The Measurement of Radiocarbon

Methods for working with and for measuring each one of the more important radioelements have been described in various texts and reports [1, 49, 93, 94]. Such detailed discussions cannot be included here. The reader must rather be referred to these reviews and to the preceding sections of this book, where methods of measurement have been described in general. We merely emphasize once again that in most of the cases, which arise in biochemistry, we use ionization chambers to measure α-rays, Geiger counters for β-particles, and Geiger counters or—better—scintillation counters for γ-rays.

Because of their great biochemical importance, exceptions will be made in the cases of carbon and hydrogen. The methods applicable to these elements have also been summarized[1, 95], and some of the procedures have been described in a book[28] and a review article [49]. We shall nevertheless present a short survey for the benefit of the biochemist. As far as radiosulfur is concerned (which is also important in biochemistry), the reader is referred to review articles[1, 93, 96, 97] and to recent experimental papers [98–101]. Phosphorus requires no special discussion, since it emits hard β-radiation.

In view of the fundamental importance of carbon in living things, it is reasonable to assume that the main part of all radiobiochemical work will eventually be carried out with long-lived radiocarbon. That this has not been the case until recently is doubtless due to the fact that radiocarbon was relatively late in being discovered and made available, and also that it presents special problems in measurement.

The difficulty in measurement is due to the unusual softness (low energy) of the β-radiation, which has a maximal energy of only 155 keV (^{24}Na: 1390, ^{32}P: 1710, ^{131}I: 608 keV). No γ-rays are emitted by ^{14}C. The thickness of a layer absorbing 50% of the β-rays is only 3 mg/cm^2, and that of a layer giving 50% self-absorption is only 6.5 mg/cm^2.

For this reason, the use of a liquid counter, for example, is out of the question in the case of radiocarbon. Counters with mica windows can be used only when the activity of the sample is so great that a poor yield can be tolerated. The yield may be of the order of 10% (with thin windows weighing $\simeq$ 2 mg/cm^2) provided that little self-absorption occurs. This will be the case, for example, if the sample consists of an evaporated drop of a dilute, but very active, solution (p. 89). To ensure even spread, agar may be added[102, 103]. Such solutions may contain the starting materials for biochemical isotope experiments. On the other hand, radiocarbon in metabolites is usually so diluted with inactive carbon that fairly thick layers of organic substances, or of the carbonate obtained from them by combustion, must be counted with the window counter. Good uniformity of the barium carbonate is obtained by mixing with a surface-active reagent[104]. (It is also possible to completely dissolve dry, bone-free animal tissues in formamide. The solvent is evaporated, and a layer of active material remains[105].) If the counting yield is assumed to be 10% in the case of thin layers, only 3% will be measured at a thickness of 10 mg/cm^2, and only 1% at 20 mg/cm^2. In many cases these yields are not sufficient.

Considerably higher counts are obtained with flow counters (p. 80) which are usually operated in the proportional region. The sample may again be either an organic compound, or the barium carbonate obtained by burning it[106, 107].

Self-absorption is entirely eliminated only when the carbon is introduced into the interior of the counter tube as a gas (p. 81). Approximately 100% of the activity can therefore be measured with gas counters. Furthermore, quite considerable quantities of radiocarbon can be introduced into such counters; this fact naturally also increases the sensitivity of the measurement. For example, 0.5 millimol CO_2 are present in 25 ml at 0.5 atm. In contrast, only 0.2 millimol of carbon can be placed beneath a mica window 2 cm in diameter, even if the barium carbonate layer has a thickness of 20 mg/cm^2 (and hence, a high self-absorption).

Counters filled with carbon dioxide can be operated in the proportional region[108–112] or in the Geiger region[113–115]. Gas Geiger tubes at 0.25–0.50 atm (and presumably also at higher pressures) with external quenching circuits give very good plateaus, which can be further improved by the addition of CS_2 vapor[116], petroleum ether, or benzene. The excellence of the plateau is hardly affected by water vapor, but is much impaired by traces of oxygen.

The method of Libby[16] (use of an internal Geiger counter, the inside wall of which is coated with elementary carbon) has never been used in biochemistry, since samples consisting of several grams of carbon are required. Despite the fine results obtained by this method, it is now abandoned even for radiocarbon dating, because the elementary carbon tends to adsorb radioactive impurities, and also because gas-counting procedures can be applied to much smaller samples.

In summary, we may say that window counters suffice for samples with

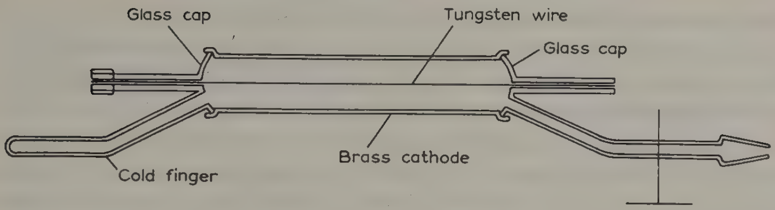


Fig. 15. Cross-section of a gas Geiger counter[113, 114].

high specific activities, but that gas counters are necessary when the specific activity is low. Flow counters occupy an intermediate position.

Only in special cases does the biochemist apply other methods, such as scintillation counting (p. 81) or photographic procedures (p. 83). The former can be employed to advantage for sensitive measurements (on samples of low activity) when the mass of the sample is very great; the latter is applicable to samples of very small mass. The reason is that the volume of the scintillator can be made very great, while that of the sample on the photographic plate can be made very small. A great deal of radioactive material is required for sensitive scintillation counting; only a modest amount for photographic track counting.

The radiocarbon may be introduced into the liquid phosphor as a soluble organic compound[117–123], for instance as a gas separated by chromatography[124], as a suspension[125, 126], or, if the phosphor is a strong base, simply as carbon dioxide[127]. Combustion is of course not necessary in the first two cases. The same is true when a thin layer of the solution is measured with a solid phosphor[128, 129], or a solution is 'mixed' with beads or filaments of phosphor[130].

The sensitivity of the various methods of measurement may be expressed either in terms of the limit of detection (in curies) or in terms of the dilution limit (curies/mol). The limit of detection refers to the amount of radiocarbon detectable, and the concentration limit to the minimum specific activity detectable. (These concepts are derived from the similar terms employed by Feigl in analytical chemistry.) The limit of detection is important when small samples are measured, and the concentration limit when the samples are large. The sensitivities of the various procedures useful for measuring radiocarbon have been compiled in tables[131].

Organic compounds can be ashed by 'dry' procedures[28, 95, 132–135]. The CO_2 and H_2O produced may be trapped separately in cooled tubes, and counted. Such dry procedures are, however, not usually chosen for radiocarbon. In the first place, they are rather cumbersome, and in the second place, radioactive substances may be retained on filling of the tubes and may contaminate subsequent samples. The great precision afforded by these methods is not required either, since the errors of sampling and counting, as well as those caused by biological variability, are in any case relatively large. Dry procedures are indispensable, however, when hydrogen must be determined in addition to the carbon.

It is otherwise preferable to employ wet ashing with acidic oxidizing agents[28, 95, 132, 134, 136–141]. The radioactive carbon dioxide is allowed to diffuse into alkali under the vacuum produced by an aspirator. However, since the speed of diffusion drops rapidly when even small amounts of air are present, and the carbon dioxide is then not completely absorbed in a reasonable period of time, it is advisable to suck the carbon dioxide through the alkali with the aid of a fritted disc. Routine combustions can be carried out rapidly in this way. The glass apparatus can be decontaminated easily and reliably with chromic acid cleaning solution, even after the combustion of very active samples.

A procedure has been reported recently, in which the dry sample is oxidized on a platinum gauze in a flask filled with oxygen, and the CO_2 is absorbed by alkali in the same flask. This procedure has advantages similar to those of the wet method[142].

Barium carbonate slowly exchanges its CO_2 with that of the air, especially when the air is warm and moist; it thus loses activity, and should therefore be kept in a desiccator if great precision is desired over long periods of time. Less care is required during periods of the order of days[95, 143].

14. The Measurement of Radiohydrogen

Tritium is also becoming more and more important in biochemistry[144]. Labeling the covalently-bonded hydrogen is often just as useful as labeling the carbon itself. Like radiocarbon, tritium can be prepared essentially free of carrier (p. 11). Since its half-life is 500 times shorter, much higher specific activity can be obtained, and radiohydrogen is much cheaper than radiocarbon of equal activity.

However, the difficulties of measuring hydrogen are even greater than those encountered with carbon[1, 28, 49]. Again, no γ-radiation is emitted, and the β-rays are much softer than those from carbon. It is therefore impossible to use a window counter. Flow counters can be used, but the measuring yield is low if the sample is at all thick[145–150]. In most cases, the tritium is introduced into the instrument in the form of a gas[144].

Measurement with ionization chambers[151–153] or proportional counters[154] has been recommended in the literature. However, because of the simpler amplifier required, a Geiger counter is often preferable. The tube can be filled with hydrogen gas[134, 155, 156]; metals may be used to liberate the hydrogen from the water obtained on burning the organic samples.

Unfortunately, the plateau is not very satisfactory. Another disadvantage of hydrogen is that it cannot be condensed and trapped by cooling.

Tritium can also be introduced into the tube in the form of water vapor although the formation of a film of water would affect the insulation. However, fairly good results have been reported with simple procedures, where the water vapor is mixed with one of the conventional filling gases. Closed counters[157–159] and flow counters[160,161] have been used.

These procedures are attended by two disadvantages. In the first place, active samples contaminate the tube by adsorption of water or exchange of hydrogen with various parts of the tube. The tritium thus taken up must be 'washed out' by repeated treatment with inactive water vapor. In the second place, the amount of water vapor which can be introduced into the tube is limited by the vapor pressure of water at the prevailing temperature; at room temperature this is only 15 mm. Both difficulties can be avoided by transferring the hydrogen from water to acetone by catalytic exchange. Acetone has a high vapor pressure and is not strongly adsorbed[162].

Very useful, but relatively complicated, are procedures in which the hydrogen is incorporated into suitable hydrocarbons. For example, the active water can be treated with calcium carbide[152,163], aluminum carbide[148,164,165], methyl[166] or butyl Grignard reagent[28,167] to give acetylene, methane, or butane. These are gases which are not adsorbed, do not affect the insulation, and give satisfactory plateaus. Acetylene and butane are also condensible.

Because of the difficulties involved in working with the Geiger counter, scintillation counting has gained in importance. Rough determinations can be done with solid samples[168]. More often, the radioactive organic compounds or the active water are mixed with the scintillator, as in the case of carbon[118,121,122,123,169–174]. Such procedures have been used, for example, to determine tritium in urine[175]. The measuring yields are not very good, and the background is high. However, with the use of an appropriate circuit hydrogen and carbon can be assayed in the presence of one another.

Because of the short range of its rays, tritium is very well suited to autoradiography, giving even better resolution than radiocarbon[175a]. Compounds of tritium in paper chromatograms are often located by autoradiography although the rays are strongly absorbed in the paper. A windowless flow counter for scanning has been constructed[176]. Measurement is made easier by soaking the paper with a liquid scintillator[177,178].

The combustion of organic compounds of tritium must naturally be performed by a dry procedure, *e.g.*, in a combustion oven (of quartz or metal)[28,134,167] or in a bomb[165].

15. Statistical Fluctuations

If the radioactivity of the samples is high, the differences in the measured activities of samples from duplicate experiments are caused primarily by the variability of the biological material, which is known to be quite large in many cases. This scattering of the measured values is in principle not different from the scattering observed in biochemical investigations by other methods, like gravimetric analysis or absorption spectrography. The situation is different when the activity to be measured is small.

According to quantum theory, one cannot, in the case of light absorption, foresee whether any given molecule which is exposed to a beam of light will absorb a quantum of energy and thus have an electron raised from the ground state to an excited state. It is only possible to ascribe a certain probability to such an occurrence. The behavior of the individual molecule is, therefore, not strictly determinate; on the contrary, when light of constant intensity strikes a sample, there will be varying intervals between the individual processes of light absorption or emission. The lengths of these intervals are distributed statistically; the individual intervals may, indeed must, be sometimes longer and sometimes shorter than the average. Yet since the absorption or emission of light can in practice be observed only when millions of molecules are absorbing (emitting), these fluctuations are not noticed. Only the average value of the interval is observed, and the average value of the absorption (emission) coefficient is derived.

The time of the disintegration of an individual atom, like that of the absorption or emission of an individual quantum of light, cannot be foreseen exactly. Hence there must be time intervals of varying lengths between the disintegrations of identical radioactive atoms. However, in contrast to the measurement of light absorption, the measurement of individual events (*i.e.*, the emission of individual radioactive rays) is possible and necessary, for example, with Geiger and scintillation counters. Each individual ray measured corresponds to the disintegration of a single atom; the intervals between rays must vary, even when they are emitted by a radioactive substance consisting of only one kind of atom. For this reason, the measurement of weakly active samples is always attended by a 'statistical error', which is the greater, the smaller the number of counts observed. This statistical variation is superimposed upon the biological variation, and predominates when samples of very low activity are measured.

Here we shall present in elementary form a few formulae, from which the magnitude of the statistical variations can be estimated[91]. An appropriate measure of the fluctuations is given by the standard deviation *S.D.*

(or m). When the number of random events is measured, the standard deviation is given by

$$m = \pm \sqrt{\frac{\sum_{1}^{n} (\bar{N} - N_i)^2}{n}}$$

where $\bar{N}$ is the arithmetic mean of all the measured values, N_i the value of the i-th measurement, and n the number of measurements. The symbol Σ indicates that the summation is to be performed over all values from 1 to n. The standard deviation is therefore the 'root mean square' of the deviations (square root of the average of the squares of the deviations). This standard deviation is a minimum value, in that it would be greater if one were to replace $\bar{N}$ by any value other than the average of the measured values.

When activities (here the numbers of counts N) are measured, the values conform to the so-called Poisson distribution; the standard deviation is then

$$m = \pm \sqrt{\bar{N}}$$

The unknown mean value $\bar{N}$ can, to a sufficient degree of approximation, be replaced by any one of the observed values N_i, since they lie close to the mean value. Table 5 gives the standard deviation and the coefficient of variation (standard deviation, expressed as a percentage of the mean, $= 100\ m/\bar{N}$) for various numbers of counts, as determined for example with a Geiger counter.

The absolute value of the standard deviation increases with the number of counts, but the percentage error (coefficient of variation) decreases. It is of course the latter which is decisive for the evaluation of an experiment. It is apparent that a result becomes more reliable as the sample is counted longer, as common sense would indicate.

Table 5 holds only on the assumption (which is made throughout this section) that the error is purely statistical in nature, no additional errors

TABLE 5

STANDARD DEVIATION AND COEFFICIENT OF VARIATION

Total counts (N)	*Standard deviation* (m)	*Coefficient of variation* (%) ($100\ m/N$)
10	3.16	31.6
100	10.0	10.0
1,000	31.6	3.2
10,000	100	1.0

being introduced, for example, by poor functioning of the counter, by variations in the thickness of the sample or by changes in the geometry.

With very weakly active samples, the background also contributes to the uncertainty in the result, for the background, which in this case constitutes an appreciable fraction of the total count recorded, is itself subject to random (statistical) fluctuations. It may, therefore, make up a larger fraction of the total count during one interval than another. The theory of errors asserts that in such a case the standard deviation of the activity of the sample P—that is, of the difference between the measured value N and the background L, referred to the same period—is given by the expression

$$m_p = \pm \sqrt{N} = \pm \sqrt{P + L}$$

provided the true mean value of L can be determined once for all by counting the background for a sufficient length of time.

However, the background should be measured anew from time to time to make sure that it has remained constant. This is not necessarily the case, if the condition of the counter or other factors have changed. If the most probable value of the background count, L, is derived from a determination of the background over a limited time, the error in this measurement contributes to the total S.D. In this case, the true counting rate per unit time, p, with its S.D., is given by

$$p = N/t_N - L/t_L \pm \sqrt{(N/t_N{}^2) + (L/t_L{}^2)}$$

where t_N and t_L are the measuring times for the sample and the background.

As an illustration, we take a series of measurements in which the specific activity of the carbon in the organs of rats, which had been fed radioactive butter yellow, was determined with the aid of a gas counter (p. 93). The carbon dioxide was obtained by a wet-ashing procedure, and exhibited only slight activity[179]. The average value of the background was assumed to be 40 c.p.m. (counts per minute). Since the samples were usually counted for 16 min, the standard deviations were as given in Table 6.

TABLE 6

STANDARD DEVIATIONS IN THE DETERMINATION OF CARBON DIOXIDE OF LOW ACTIVITY

True counting rate	*Standard deviation per unit time*
1	± 1.6
2	± 1.6
5	± 1.7
10	± 1.8
100	± 3.0

It can be shown that the probability for the error of a single measurement to exceed the standard deviation is approximately 32% provided the activity is not too small. Twice the standard deviation is exceeded, on the average, in 5% of the cases, three times the standard deviation in 0.3% of the cases. Fig. 16 gives the complete probability distribution of the errors of individual measurements; it is the well-known Gauss curve.

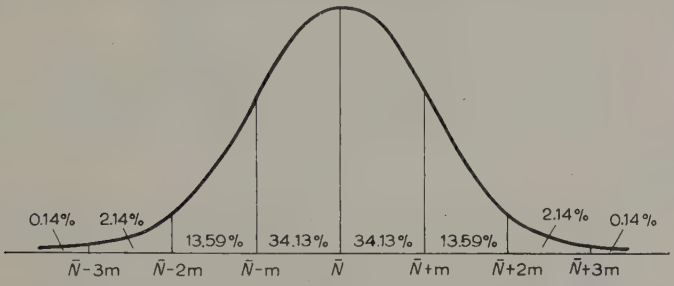


Fig. 16. Gaussian distribution of errors. (The percentage figures give the probability that an observation lies within the given region. $\bar{N}$: arithmetic mean of all observations, m: standard deviation).

In one case in the author's laboratory, a CO_2 sample counted with the gas counter (background: 40 c.p.m.) was found to have an activity of 2.50 ± 0.14 c.p.m. In order to minimize the statistical error in measuring an activity so small, it was necessary to count for nearly a week, the counter being filled alternately with the active sample and with inactive CO_2[180].

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CHAPTER IX

THE APPLICATION OF RADIOACTIVITY TO THE ANALYSIS OF LIVING MATTER

1. General Survey

In this chapter we shall temporarily disregard the fact that the constituents of living matter are in a state of constant change. We shall explain instead how radioactivity can be used to study the chemical composition of a sample at any given moment. That is, we shall make a sort of analytical snapshot; the state observed may be either a stationary or a changing one. The constituents with which we are concerned may be quite varied; in the simplest cases they are inorganic ions, but radioactivity may be employed as well for the analysis of complicated organic compounds, including even proteins or nucleic acids.

The application of radioactivity to all branches of analytical chemistry has assumed a rapidly growing importance; a comprehensive survey and reviews of newer developments[2–5] have recently appeared. Analytical chemistry exploits the special advantages of techniques employing labeled atoms, *viz.*, sensitivity of detection, rapidity and simplicity of measurement, in certain cases non-destructiveness, and finally, the possibility of distinguishing between labeled and unlabeled, but chemically identical, atoms or molecules. On the other hand, the precision of radiometric analysis is only moderate, since the measurements of activity are usually reproducible only to within one or two per cent[6].

The great sensitivity with which radioactive substances can be detected by their activity was recognized in the early days of the study of radioactivity. The short-lived naturally-radioactive elements cannot be assayed in any other practicable way. It was mentioned in the first chapter of this book that the content of certain short-lived radioelements in living matter was determined a long time ago by means of activity measurements.

By mixing inactive elements with their active isotopes, it is possible to 'label' them, or mark them, with an 'indicator'. If the specific activity—

that is, the ratio of the numbers of active and inactive atoms—is known, the amount of labeled element in the sample can be deduced from the activity measurement. Particularly important is the case where the specific activity is the same in all parts of the system. For instance, in the determination of elements, these are added to the system before the process under investigation starts. The elements are added in labeled form, and at known specific activity, and subsequent changes in the specific activity (by dilution with inactive atoms) must be avoided. Thereafter, any desired number of sensitive determinations can be carried out without re-determination of the specific activity. This method is known as 'indicator analysis', and is discussed in more detail in Section *2* of this chapter.

Before the discovery of artificial radioactivity, the number of radioelements useful as indicators was small, and the field of application of the method correspondingly limited. In order to extend the advantages of activity measurements to include the determination of inactive elements, the radioelements were employed as 'reagents'. Although useful radioisotopes of most elements are now available, and the original reasons for using radioelements as reagents are no longer compelling, the 'radioreagent' methods continue to prove valuable for various analytical purposes (Section *3*).

The main reason for the lack of precision in biochemical analyses often lies in the fact that the material to be determined has to be separated from a mixture of similar substances. Thus often either low purity or low yields must be accepted. This source of error obviously disappears if the pure substance can be isolated in a known yield. The yield can be determined with the aid of labeled atoms by the well-known 'isotope dilution method', which will be described in Section *4*.

An especially sensitive and simple determination of many elements can be carried out by 'activation analysis'. This method, in contrast to the procedures mentioned above, does not make use of radioelements present before the start of the analysis. The sample is exposed to a current of elementary particles, usually slow neutrons, in order to transmute the element to be determined. Other conditions being constant, the resulting radioactivity is proportional to the amount of the element; hence the analysis can be based on a measurement of the activity. In a variant of this procedure, no radioactive nuclei are produced, but the transmutations occurring during the irradiation are observed. Activation analysis is discussed in Section *5*.

In a further method, the absorption of a beam of particles (in practice, of neutrons) passing through the sample is measured. If the element to be determined absorbs the particles much better than the other elements of the sample, the quantity present may be estimated ('absorption analysis'; Section *6*).

2. Indicator Analysis

The very great sensitivity with which radioactive substances can be detected makes it possible to analyze for the merest traces of labeled materials. Many examples of indicator analysis, most of them taken from fields other than biochemistry, are given in the reviews mentioned.

If more than one radioelement is present, or if labeled molecules are to be determined, the 'radiochemical purity' of the sample is of decisive importance. It must be confirmed that the specific activity remains constant on further purification. "Blind adherence to rule-of-thumb purification procedures, inflexibility in devising analytical methods and failure to make adequate control experiments are an invitation to disaster. Tracer techniques badly exploited can result in mistakes of a magnitude hardly possible with conventional chemical procedures. In view of the rapidly increasing availability of the radioactive tracers, it is important to emphasize that there is no substitute for good chemistry"[7].

As a typical example of a piece of research based upon indicator analysis we cite the study of the absorption of arsenic by the organs of the cotton rat[8]. The rats were injected intraperitoneally with 1.6 mg of As per kg of body weight, in the form of radioactive sodium arsenite, and killed 24 h afterwards. It was easy to determine as little as a few tenths of a microgram of arsenic per gram of whole tissue by measuring the activity.

The daily requirement of a cow for cobalt is of the order of 1 mg. Traditional methods of analysis are useless for the study of the cobalt metabolism and the distribution of cobalt in the organs, if physiological conditions are to be maintained. The problem can be resolved easily, however, with the use of radiocobalt[9]. The studies on the absorption of elements, reported in Chapter X, may also serve to show the power of the indicator method.

The assumption that the specific activity of the element is constant, and that the quantity of the element is therefore determined unambiguously through its activity, is not always valid. The specific activity of elements which occur in the body must depend upon the time and place until a stationary state has been reached; a good example would be iodine. The same thing would hold true in the determination of molecules instead of atoms or simple ions. As will be discussed in more detail later—particularly in connection with photosynthesis—the time required for the various organic compounds in living matter to reach their final specific activities depends upon the role which they play in metabolism.

Indicator analysis can also be used to test and to improve other—often time-honored—methods of analysis; indeed it has now become an indispensable aid for this purpose. Indicator analysis is the simplest and best way, for example, of finding out whether separations are quantitative, what happens to impurities, whether losses occur on washing precipitates or solvent phases. Many examples will be found in the literature quoted.

An extensive study of the separation of trace amounts of beryllium from biological materials has been carried out with the help of ^{7}Be[10]. Since this radioelement can be obtained in a carrier-free state, the detection of the element by its radiation is exceptionally sensitive. Appreciable losses of beryllium during ashing were observed in early studies; it was now possible to demonstrate that these losses were not due to volatilization of the element, but to the formation of sparsely-soluble oxides; the latter could be brought into solution only by long treatment with concentrated sulfuric acid. On the other hand, in the evaporation of benzene solutions of beryllium acetylacetonate, material is certainly lost by volatilization. Other authors have studied the coprecipitation of labeled RNA with DNA[11]. Radioiodine was used to find the sources of error in the determination of iodine in blood[12].

Important errors can be caused by adsorption on the walls and on filtering media (Chapter III). Cations are particularly easily taken up by ion exchange in glass and in paper. Experiments with radiolead showed that noticeable losses occur even at lead concentrations as high as 10^{-3} M[13]. Such losses can be minimized by the addition of other cations. The concentrations required depend upon the strength of the adsorption of the added ions. For instance, hydrogen ions, at concentrations as low as 5×10^{-3} M, decrease the loss of lead on filter paper or glass, although fair amounts of lead are lost from alkali chloride solutions as concentrated as 1 M. Analogous experiments have been carried out with other radioions[14–16], and with labeled proteins[17]. Adsorption can also be reduced by coating the walls with various synthetic products[18].

Indicator analysis is also helpful in disclosing a compensation of errors in some seemingly correct methods. Good results were obtained, for example, in the analysis of sulfate as 4-chloro-4′-aminodiphenylsulfate. Investigations with radiosulfur revealed that the 'success' of the method was due not to the exactness of its stoichiometry, but to a compensation of errors; on the one hand, some reagent is coprecipitated, but, on the other hand, a portion of the sulfate is not precipitated, or is lost on washing the precipitate[19].

Very sensitive and selective analyses can be carried out by combining chromatography and indicator analysis; both column and paper chromatogaphy can be used (*cf.* p. 20).

For example, the radioactive amino acids obtained on hydrolysis of the protein of carbon-labeled tobacco mosaic virus were separated by column chromatography. Half of each fraction of the eluate was used for colorimetric analysis. The remaining portions, each containing a single amino acid, were combined, ashed, and counted. In this way the amount, total activity, and specific activity of each amino acid were determined[20]. A further example is the separation of labeled phosphate esters (hexose monophosphate, hexose diphosphate, ribulose diphosphate, phosphoglycerate) from extracts of algae on Dowex anion exchangers[21].

Whereas the quantitative paper chromatographic separation of isomeric amino acids (*e.g.*, valine–norvaline) is difficult, these substances can be determined in the presence of one another with the aid of radioindicators. One of the substances (valine, for instance) is added to the sample in labeled form, but in unweighable quantity, and the mixture subjected to paper chromatography. The spot on the paper containing the two substances is cut into narrow strips, perpendicular to the direction of migration. The quantity and activity of the amino acids in each strip is determined, and the labeling indicates which part of the spot is due to valine (maximal specific activity) and which to norvaline (specific activity, zero).

Another example of paper radiochromatography is the analysis of thyroid extracts from rats injected with radioiodine. Ninhydrin revealed the presence of tyrosin and di-iodotyrosin on the strip, but between these two spots there was a radioactive region where ninhydrin did not give a color reaction. When synthetic mono-iodotyrosine was added to the extract, however, a spot appeared at this point. The thyroid obviously synthesizes small, but relatively active, quantities of mono-iodotyrosine. This compound may be an intermediate in the formation of di-iodotyrosine[22–24]. Other ^{131}I compounds appearing in the blood of mammals such as rats, sheep, and horses, after the administration of iodide or organic compounds of iodine were also separated and analysed by paper chromatography [25,26]. Paper chromatography proved suitable for establishing the chemical form in which the sulfur of labeled BAL was excreted in urine[27].

The sensitivity and simplicity of determinations with radioactive indicators has made the development of new chromatographic procedures much easier[1]. One example is the separation of the isomers of diethylethylmercaptoethyl thiophosphate (a systemic poison used as an insecticide) by partition chromatography on silica columns after labeling the phosphorus[28]. Labeled compounds have also been used to develop and test procedures which could be applied to the analysis of inactive DDT-derivatives[29] or sterols[30].

Paper electrophoresis is, of course, employed as well in indicator analysis (p. 23). When very small quantities of labeled proteins are applied to the paper, there is a danger that they may be held back on the paper by adsorption. It has been reported, however, that this adsorption can be suppressed by the addition of a suitable foreign protein. For example, even 10^{-9} g of radioactive guinea pig serum albumin (in a $10^{-5}\%$ solution) migrates with its characteristic velocity if a relatively large quantity of normal human serum protein is added[31]. Good sensitivity in the detection of carbon-labeled substances is obtained by ashing the pherogram strip-by-strip, and introducing the carbon dioxide into a gas counter. This procedure has been used in the analysis of labeled serum proteins; in this case, electrophoresis on starch has been employed[32, 33].

3. Methods Employing Radioactive Reagents

The procedures, which originated before the discovery of artificial radioactivity, all used radiolead as the active reagent[34]. An example is the determination of chromium. The chromate solution is treated with an excess of radiolead (thorium B) solution of known concentration and activity, the precipitated lead chromate is filtered off, and the activity of the filtrate determined. From the activities of the filtrate and of the original lead solution, the amount of lead precipitated can be calculated and hence the amount of chromate present can be evaluated[35]. Proteins can be determined by precipitation with radioactive tungstate[36].

Procedures with radioreagents can be based on reactions other than the formation of precipitates. Many methods have been based on the formation of complexes[37]. Water can be determined by reaction with aluminum chloride labeled with ^{36}Cl and measurement of the activity of the HCl produced[38].

Iodine can be assayed by oxidizing it to iodate with bromine and subsequently adding excess radioactive iodine. Acidification causes the formation of elementary iodine, which is extracted with a solvent; from the radioactivity of the iodine the amount of iodine originally present can be calculated[39].

Sterols can be treated with *p*-^{131}I-benzoic acid, the reaction products separated by chromatography, and the amounts of the individual sterols determined from the radioactivities of the various fractions[40–42]. When a number of similar substances are present, it is advantageous to combine the use of radioactive reagents with the isotope dilution method, which will be discussed in the next section.

The number of a particular kind of functional groups present in a pure substance of known molecular weight can be found by the use of active reagents. Labeled acetic anhydride was used to show that the aldosterone molecule contains two hydroxyl groups[43]. The same reagent is suitable for amino groups (p. 114). Hydroxyl and amino groups have also been determined with 4-methoxy-3-^{36}Cl-benzoic acid[44] and with *p*-^{131}I-benzoyl chloride[41].

^{131}I-iodine monobromide is employed for the micro-determination of the iodine numbers of fatty acids. A known amount of solution of fat is introduced onto paper, and the solvent evaporated off. The spot is treated with the active reagent, and the excess reagent washed out. The activity remaining on the paper, together with the specific activity of the reagent, give the required iodine numbers[45–47]. For the determination of saturated fatty acids on paper chromatograms the acids may be converted into the silver salts, and these salts subsequently treated with radioactive iodine[46, 47].

The molecular weight of polysaccharides with a reducing terminal group can be determined with a radioreagent. The polysaccharide is treated with radioactive sodium cyanide of known specific activity, and the cyanhydrin hydrolyzed to a carboxylic acid, whose specific activity depends on the molecular weight[48, 49].

Procedures have recently been described which involve an interesting combination of radioreagent methods with animal experimentation. Without doubt these proceduces will find many applications in biochemistry in the future.

The determination of thiouracil in animal organs may serve as the first example[50]. The organs were fed to rats, thus affecting the capacity of the thyroids of the rats to take up radioiodine. It was therefore possible to derive the amount of thiouracil from the radioactivity of the thyroids after the administration of radioiodine. The results were evaluated with the aid of 'calibration' animals.

Hormonally-active substances, which stimulate the thyroid, are assayed by feeding them to chickens which have previously received radioiodine. The excretion of the radioiodine then serves as a measure of the stimulant [51]. Parathyroid hormone was determined by the increase in excretion of radiophosphorus by rats whose parathyroids had been removed[52], and the 'intrinsic factor', which is active against pernicious anemia, by the uptake of cobalt-labeled vitamin B_{12}[53].

Deoxycorticosterone is measured by its effect in speeding the urinary excretion of potassium (^{42}K)[54]. The ratio of sodium (^{24}Na) and potassium (^{42}K) in the urine of adrenalectomized rats is altered by aldosterone[55]. The analysis of the hormone was accomplished by measuring the alkali metals in the urine, first without an absorber, and subsequently with an interposed absorber of 300 mg/cm^2 thickness.

The absorber decreases the intensity of the β-rays from potassium by a factor of only 3, but of those from sodium, by a factor of 20. The data were computed on the basis of a calibration curve, which gives the quantity of aldosterone (or the logarithm of the quantity) directly as a function of the ratio of the two activity measurements (or the logarithm of the ratio) (see also p. 119). Aldosterone proved to be 120 times more effective than deoxycorticosterone.

Vitamin determinations can be based on the fact that the rate of uptake of labeled phosphate by the bones of intact rats depends upon the vitamin D content of the diet[57]; the uptake of radiophosphorus by yeast depends on the pyridoxine or pantothenic acid content of the medium[58].

Alkaline phosphatase can be determined quantitatively in histochemical work by precipitating the liberated phosphate *in situ* with radiocalcium and measuring the activity after washing out the excess reagent[59]. More examples of the applications of tracers to quantitative staining reactions have been reported[60, 61].

4. Isotope Dilution Methods

When separations are not quantitative, the yield can be determined by the method of isotope dilution. This makes it possible to improve the accuracy of many analyses quite considerably, notwithstanding the fact that the radioactivity itself (as has been emphasized previously) is not measured very exactly. It is precisely in the biochemical field, where quantitative separations are often unobtainable, that these procedures have attained much importance[62].

The principle of the 'simple' isotope dilution method is the following. Some of the material to be determined is added to the mixture being analyzed. The added material is labeled, and both its amount and its activity are known. After thorough mixing of the sample, the substance to be assayed is isolated by some suitable method, and submitted to whatever procedures are required to obtain a pure preparation. The yield is not of primary importance. From the activity of the pure substance the yield, and hence the quantity of the material in the original mixture are calculated.

If the unknown quantity of material is denoted by M_1, the quantity and specific activity of the added labeled material by M_2 and A_2, the specific activity of the isolated (pure) substance by A_3, then

$$A_3 \doteq A_2M_2/(M_1 + M_2), \text{ or } M_1 = M_2([A_2/A_3] - 1)$$

If the labeled material is free of carrier, and has, therefore, a very high specific activity, M_2 is negligible compared to M_1, and we have

$$M_1 = A_2M_2/A_3 = G_2M_3/G_3$$

where G_2 and G_3 are the activities of the added and of the isolated material. In other words, if carrier-free material is added, the activity ratio G_3/G_2 directly gives the yield, as is obvious.

The isotope dilution method is based upon a study by Hevesy[63]. Variable results were obtained when micro-quantities of lead were determined by deposition of lead dioxide; but after addition of a known amount of radiolead and measurement of the activity of the deposit it was possible to determine the yield and the total quantity of lead present.

Isotope dilution methods were introduced into biochemistry in the laboratories of Schoenheimer and Rittenberg[64–66] and of Ussing[67]. One of the first applications was to the determination of individual amino acids[68]. If a known quantity of the labeled acid, *e.g.*, glutamic acid[69, 70], is added to a mixture, and the acid is isolated in pure form, the yield is obtained by comparing the activities of the added and of the isolated substances. Procedures for separating pure amino acids for this purpose have been developed[71].

When amino acids—and also other constituents of living matter—are to be determined, and an optically active reagent is used, one of the optical isomers may be isolated in completely pure form, since quantitative yields are not required in the isotope dilution method. The separate determination of the two isomers is therefore possible.

Proteins are often difficult to isolate quantitatively from tissues or to crystallize; they can, however, be specifically precipitated by antibodies (Chapter XVI[72, 73]). This technique has been employed, in connection with isotope dilution, in the study of the composition of the extracellular fluid of muscle[74] and in the investigation of protein synthesis in tissue slices[75].

In biochemical technology the isotope dilution method has been used to determine penicillin G (benzyl penicillin) in impure solutions, from which the pure substance could not be isolated quantitatively. Carbon-labeled penicillin G was added, the penicillin G separated, and the content of the solution calculated from the yield and the activity[76]. Other workers have used sulfur-labeled penicillin[77]. Similar analyses have been performed for vitamin B_{12}[62, 78, 79] (separation by column chromatography), vitamin D[80], the γ-isomer of chlorocyclohexane[81–83], sterols[84–87] and gibberellic acid[88] in complicated mixtures. Glucose has been determined in hydrolysates of starch[89], choline and cholamine in mixtures of phosphatides[90], purines have been assayed in nucleic acids[91], and substituted phenoxyacetic acids in weed-killers[92].

Isotope dilution methods improve the accuracy of an analysis, but not its sensitivity. In the most favorable cases, the sensitivity is equal to that of the method employed in conjunction with isotope dilution, *e.g.*, to that of gravimetric or colorimetric determinations. The limit of detection is, however, often less favorable when mixtures of similar substances are involved, because a certain minimum quantity $M_1 + M_2$ of material is required for the necessary separations. In principle it would of course be possible to introduce the needed quantity in the form of the labeled material added, *i.e.*, to make M_2 greater than M_1. However, this would make A_3 nearly as large as A_2, and since the activity measurement is always attended with some error, the determination becomes less and less reliable as the ratio M_1/M_2 decreases, eventually becoming quite worthless.

The situation can sometimes be improved by using the 'reverse isotope dilution method'. In its simplest variant, the element or compound to be determined is applied in a labeled form from the start. The yield in the recovery from the sample at the end of the experiment is determined by adding a known quantity of the non-labeled element or compound before extraction, and measuring, by standard methods, the yield in the recovery of the latter.

An example is provided by the study of trace elements in soils and in vegetation. It is known, for example, that cobalt plays an important role in the nutrition of ruminants. It is of interest to determine what fraction of the cobalt added to the soil remains, after a given period, in the soil layers which are involved in nutrition of the animals. This question can be answered very nicely by the use of radioactive cobalt. The radiocobalt cannot be determined directly in the soil because of the strong self-absorption of the radiation. It is necessary, therefore, first to concentrate the cobalt from a large quantity of soil. However, quantitative recovery of small amounts of the element from large amounts of earth is not possible. A fair amount of inactive cobalt is therefore added to the soil sample. The sample is homogenized, a certain amount of cobalt is isolated as a pure compound, and the activity determined. The ratio of the weights of added and recovered cobalt gives the yield, and the total activity and the quantity of the cobalt remaining in the soil can be calculated from the measured activity.

The antivitamin dicoumarol was measured by reverse isotope dilution in animal tissues after the animal had been fed with the labeled compound [93]. After the death of the animal, the organs were homogenized with the addition of inactive dicoumarol as carrier, the dicoumarol was isolated and purified by recrystallization, and its activity was measured.

The distribution and chemical state of radiocarbon after application of the labeled carcinogen dibenzanthracene to the skin of mice was determined in a similar way [94, 95] (p. 310). The suspected metabolic products were added to the organs in unlabeled form, isolated after homogenization, and the yields and activities determined. A further example is the determination of the degradation products of labeled histamine in the urine of experimental animals [96].

Very frequently, however, the system under study is not under the complete control of the investigator before analysis. For example, metabolites in animal tissue, which are produced from unknown precursors, are to be determined. In this case, the reverse isotope dilution method can be combined with the radioreagent method. A labeled reagent of high specific activity is added to the mixture, and the substance to be determined is converted into a radioactive derivative. After removing the excess reagent, the same derivative is added in inactive form and the total amount of the derivative is isolated in a pure state. The quantity of the substance in the original sample is obtained from the specific activity of the derivative according to the formula

$$M_1 = M_2/([A_1/A_2] - 1)$$

where A_1 is the specific activity of the derivative, and can be calculated from that of the labeled reagent. Procedures of this type can be employed advantageously for the determination of the merest traces of material.

As an interesting example, of which the principles but not the details can be given here, we mention the analysis of mixtures of amino acids from protein hydrolysates with the radioreagent 'pipsyl chloride', *i.e.* *p*-^{131}I-phenylsulfonyl chloride[1, 97–99]. This reagent forms derivatives with all amino acids; after addition of the corresponding inactive derivatives, they are worked up by the isotope dilution method. The analyses require the determination of the amounts and activities of the isolated pipsyl derivative of the individual amino acids. The separations are best performed by paper chromatography. Analyses of labeled histamine[100] and pyrimidines[101] have also been carried out with the pipsyl reagent.

The reverse isotope dilution-radioreagent method, as explained, can be used only when the compound to be determined reacts quantitatively with the reagent. Even if it does not, a variant of the method can be applied. The compound to be determined is added to the sample in a form which contains a different label (for example, ^{35}S). After reaction with the labeled (^{131}I) reagent, the unlabeled derivative is added, and the pure derivative is isolated. The amount of material present in the original mixture can be calculated from the amount of both radioelements in the isolated derivative. By using this modification of the method, all gravimetric or colorimetric determinations are avoided, and only activity measurements (on each radioelement separately) are needed. One percent of an amino acid in 1–2 mg of protein hydrolysate, *i.e.*, 10 μg of an individual amino acid, has been determined by the pipsyl procedure[102–104].

Acetic anhydride labeled in the carbon or in the hydrogen (p. 109) is another radioreagent for the determination of amines. This reagent is also suitable for determining alcohols. Double labeling is, as in the case of the pipsyl reagent, advantageous. Very small quantities of aldosterone and hydrocortisone[43] or of amines[105] have been determined. Carboxylic acids and their chlorides and anhydrides have been assayed in the form of their compounds with p-^{36}Cl-aniline[106].

The reverse isotope dilution method is also employed in conjunction with activation analysis.

5. Activation Analysis

Activation analysis[107–111] is based on the following principle: the sample is exposed to a beam of elementary particles, usually slow neutrons, which transmute the element to be determined and produce a radioactive nuclide. This activity produced is a measure of the number of the nuclei involved in the transmutation, and hence, since the naturally-occurring elements almost always have a constant isotopic composition, of the quantity of the element itself.

Activation analysis is particularly easy when the activity produced in the sample can be measured directly, without concentration by chemical procedures. This is of course possible only when the radioelements are produced in such quantities that the self-absorption in the sample is not prohibitive.

Non-destructive activity measurement is especially favorable when the element to be determined, but none of the others, can be converted to a radioelement in good yield. Even when other elements are activated, however, a direct measurement of the activity produced in the sample is often possible.

For example, radioelements such as sodium and potassium (p. 110), which emit β-particles of widely different energies, can be distinguished by the use of absorbing foils. Other methods are based on the differences between the half-lives. When mixtures of radioelements with very different half-lives are involved, the decay curve indicates the fractions of the total activity due to the individual radioelements. The half-life of ^{32}P is 14.2 days, for example, and that of ^{76}As, 26.5 h; both are produced by bombardment with slow neutrons. Phosphorus is determined in the presence of arsenic by allowing the activity of the arsenic to decay before measurement.

Finally, it is possible to suppress the formation of unwanted radioelements by suitable selection of the conditions of exposure to radiation. The activity of every radioelement attains saturation only after exposure for several half-lives. Thus the time of exposure is so chosen that saturation is attained to a large extent for the desired, short-lived radioelements, whereas no appreciable activity of the unwanted, long-lived radioelements is induced. In order to determine arsenic in the presence of phosphorus, the irradation time is kept short, and vice versa.

When it is impossible to determine the activity of the radioelement with an intact sample, chemical separations become necessary. Certain features of these separations contribute to the advantages of activation analysis. Inactive carriers of all the elements being determined can be added to the irradiated sample. The chemical separation can then be carried out with larger amounts of material, and the difficulties of working with micro-quantities (and especially, the danger of losses by adsorption) can thus be avoided. Nor is it necessary for the separation to be quantitative; if the amount of carrier is large, compared to the amount of material to be determined, the ratio of carrier introduced to that recovered permits a calculation of the yield, and hence the total activity may be determined from the measured activity (principle of reverse isotope dilution, see p. 114). Contamination of the reagents with the material being determined does not matter either since the reagents are introduced only after the irradiation.

Activation analysis is almost always carried out by relative measurements; reference samples of known composition are bombarded under conditions as similar as possible—preferably simultaneously with the samples being analyzed. The amount of the element in the sample being analyzed can be determined from the ratio of the activities produced in the two samples.

There are several reasons why neutrons are usually employed in activation analysis. Firstly, they react with atomic nuclei much more easily than do charged particles, because they are not subject to electrostatic repulsion by the nuclei. Secondly, because they show practically no interaction with the electronic shells of the atoms they penetrate easily through many kinds of matter; transmutation is therefore not limited to the surface of the sample. Thirdly, in contrast to irradiation with charged particles, neutron bombardment does not raise the temperature of the sample. Fourthly, it is much easier in practice to work with neutrons than with charged particles.

As far as the energy of the neutrons is concerned, thermal neutrons are preferable for activation analysis. As has been remarked on p. 9, slow neutrons are most effective in causing nuclear reactions, and most specific in their interaction with various chemical elements. Another important point is that thermal neutrons undergo no change in their average energy as a result of their interaction with matter (collisions with atoms); the energy of the neutrons is therefore nearly the same throughout the sample, and largely independent of its size, shape, and chemical composition.

It is simplest to work with natural sources of neutrons. It is an important advantage that the neutron flux exhibits no irregular fluctuations.

Therefore in a series of activation analyses, standard samples need to be activated only occasionally, in contrast to analyses involving artificial neutron sources (accelerators or piles) in which it is necessary to run a standard sample simultaneously with each sample to be analyzed. The disadvantage of natural sources is again, as in the production of radioelements, their low intensity (p. 10).

A simple arrangement for activation analyses with natural neutron sources consists of a neutron source placed in the center of a block of paraffin wax. The moderator slows the neutrons down and scatters them in all directions, so that the whole block becomes filled with a sort of 'neutron gas'. The flux of slow neutrons is greatest at the source, because the neutrons diffuse back from all directions after being slowed down. In the neighborhood of the source there are, of course, beside the slow neutrons many fast neutrons, which can under some circumstances produce unwanted nuclear reactions. If no such reactions are to be feared, the sample is placed at the source; otherwise, a few centimeters of the moderator are left between the source and the sample. However, an appreciable fast neutron flux exists throughout the block. Therefore, to exclude the action of the fast neutrons, the activation of the sample is determined with and without cadmium shielding. Cadmium selectively absorbs thermal neutrons; hence the 'cadmium difference' is a measure of the effect of the thermal neutrons[112].

Most piles contain so much moderator that the slow neutrons predominate. Their greatest flux is inside the reactor. However, if the fast neutrons interfere excessively by producing undesired nuclear reactions, the sample may be placed outside the reactor, and extra layers of moderator, so-called thermal columns, may be inserted between the reactor and the sample in order to slow down the fast neutrons which escape from the pile. Ordinary water, heavy water or graphite may be used as moderators. Cadmium differences can of course be measured in the case of piles as well.

For quantitative values of the sensitivity of activation analysis we must refer the reader to the tables given in the literature[107–111]. A discussion of sources of error will also be found there[113]. Analytical procedures to be followed after activation of a complex mixture with slow neutrons have been suggested[4].

Here we merely list, in the order of their atomic numbers, the elements most important in biochemical activation analyses (Table 7). The table is idealized in so far as account is taken only of the activation reached at saturation (or after several days of bombardment), but not of the nature of the radiation emitted, the possibility of interfering side reactions, or the ease of chemical separation. The figures given for the limit of detec-

TABLE 7

SENSITIVITY OF ACTIVATION ANALYSIS

Element and atomic number	*Radioisotope produced*		*Limit of detection, in grams, after bombardment for*	
	Mass	*Half-life*	*1 day*	*to saturation*
11 Na	24	15 h	4×10^{-10}	3×10^{-10}
13 Al	28	2.3 min	7×10^{-10}	7×10^{-10}
15 P	32	14 d	1×10^{-8}	7×10^{-10}
17 Cl	38	37 min	1×10^{-9}	1×10^{-9}
19 K	42	12.4 h	4×10^{-9}	3×10^{-9}
20 Ca	45	152 d	3×10^{-6}	2×10^{-8}
26 Fe	59	46 d	1×10^{-5}	3×10^{-7}
29 Cu	64	12.8 h	3×10^{-10}	2×10^{-10}
30 Zn	69	57 min	2×10^{-9}	2×10^{-9}
33 As	76	1.11 d	2×10^{-10}	9×10^{-11}
35 Br	80	4.54 h	3×10^{-10}	3×10^{-10}
38 Sr	87	2.80 h	1×10^{-8}	1×10^{-8}
47 Ag	108	2.44 min	2×10^{-11}	2×10^{-11}
51 Sb	122	2.63 d	6×10^{-10}	2×10^{-10}
53 J	128	25 min	1×10^{-10}	1×10^{-10}
79 Au	198	2.69 d	4×10^{-11}	1×10^{-11}

tion refer to a thermal neutron flux of 10^{12} cm^{-2} sec^{-1}. If the flux is smaller, the limit of detection increases proportionally. The flux in the Harwell reactor, which is used for activation, ranges from 10^{11}–10^{12}, depending on the location inside the pile.

We shall now present several examples to illustrate the possibilities of applying activation analysis to biochemical problems.

The detection of arsenic in hair, a problem in forensic medicine and in industrial hygiene, can be accomplished without destroying the sample [114, 115]. After the activation of individual hairs in the reactor it was possible not only to measure the total amount of arsenic present but even to determine the location of the arsenic by scanning the hair with a counter, and thus to decide over what period of time the arsenic had been introduced [116]. Other workers have added inactive arsenic salts as carriers after the activation of biological samples (hair, nails, internal organs) and then performed chemical separations. In suitable circumstances as little as 10^{-10} g of As could be detected in a drop of urine or of blood [117]. Strontium in bone has also been determined by activation [118].

In order to determine sodium and potassium in the presence of one another, without chemical separation, advantage is taken of the difference in the energy of the β-rays, since the half-lives are similar [119–121]. For example, the radiation is first passed through a filter 700 mg/cm^2 thick, which is transparent to the γ-rays of both elements, but partially opaque to the β-particles of potassium and completely opaque to the β-particles of sodium. A second measurement is made through an absorber of 1700 mg/cm^2, which permits only γ-rays to pass. This procedure has made it possible to determine potassium (down to 3 μg) and sodium (to 0.3 μg) in the nerve fibers of the squid. The standard samples consisted of alkali carbonates (see also p. 111).

Another important procedure is the activation of paper chromatograms by neutrons, followed by scanning with a counter, or by autoradiography. Many substances of biological interest contain atoms, such as halogen[121a] or phosphorus atoms, which are easily activated by slow neutrons from the pile[122, 123].

Activation by fast neutrons can be used to detect small amounts of phosphorus, sulfur, and chlorine on paper chromatograms. The paper strips are placed in a hollow tube of beryllium, which is exposed to fast deuterons in a cyclotron; the reaction $^{9}Be\ (d, n)\ ^{10}B$ produces fast neutrons, which, in turn, cause the following nuclear reactions: $^{31}P\ (n, p)\ ^{31}Si$, $^{32}S\ (n, p)\ ^{32}P$, and $^{35}Cl\ (n, p)\ ^{35}S$. (Only the last of the three can occur with slow neutrons.) The activities produced can be distinguished by their different half-lives and energies[124]. The extent of interference by the side reactions $^{31}P\ (n, \gamma)\ ^{32}P$ and $^{34}S\ (n, \gamma)\ ^{35}S$, which do not occur with large yields except with slow neutrons, must be estimated from the ratio of the fluxes of fast and slow neutrons.

The specific activity of small amounts of adenosine triphosphate (ATP) on a paper chromatogram was determined in an elegant way during the course of an investigation of the action of insecticides[125]. The ATP was obtained from house flies which had been treated with the poison, methyl bromide, and injected with labeled phosphate. After the radiophosphorus in the ATP had been measured and had decayed, the chromatogram was activated with slow neutrons and measured again to obtain the total amount of ATP-phosphorus. A comparison of the two activities gave the specific activity of the ATP. This permitted the calculation of the fraction of the phosphorus in ATP which was derived from the injected phosphorus. (The paper chromatographic distribution of the injected radiophosphorus has already been presented in Fig. 3 on p. 23.)

When a radioelement produced by activation is not isotopic with any of the inactive elements originally present in the sample (not even with the element from which it is produced), it can be concentrated from a large sample, and thus isolated chemically as a substance with high specific activity (p. 12). This increases the sensitivity of the determination enormously. Such concentration is in principle possible, for example, in the cyclotron work described above with the radioelements chlorine, phosphorus, and sulfur. Minute quantities of nitrogen can be detected by the reaction $^{14}_{7}N$ (n, p) $^{14}_{6}C$ with slow neutrons; a large excess of inactive carbon in the sample does not interfere, because the reaction $^{13}_{6}C$ (n, γ) $^{14}_{6}C$ occurs only to a slight extent, *i.e.*, with a small effective cross-section[126].

A sensitive determination of uranium, also in biological samples, can be performed by fission with slow neutrons, followed by isolation and measurement of one of the fission products. Because of their appropriate half-lives and chemical behavior, the fission products ^{131}I, ^{132}Te and ^{140}Ba are suitable[1].

Among the nuclear reactions which have been proposed for analytical purposes, and which are brought about by particles other than neutrons, we note the following: $^{14}_{7}N$ (d, n) $^{15}_{8}O$ ($\tau = 2$ min) for the determination of nitrogen[127], and $^{23}_{11}Na$ (d, p) $^{24}_{11}Na$[128, 129] and $^{54}_{26}Fe$ (d, n) $^{55}_{27}Co$[130] for the determination of sodium and iron. Oxygen can be measured through the reaction $^{16}_{8}O$ (t, n) $^{18}_{9}F$ ($\tau = 1.9$ h); the required fast tritium ions can be produced indirectly in the pile from the reaction $^{6}_{3}Li$ (n, t) $^{4}_{2}He$ (p. 11)[131]. The reaction $^{18}_{8}O$ (p, n) $^{18}_{9}F$ has been applied to the study of photosynthesis [132] (p. 173).

The reactions $^{16}_{8}O$ (γ, n) $^{15}_{8}O$ ($\tau = 2$ min), $^{12}_{6}C$ (γ, n) $^{11}_{6}C$ ($\tau = 20$ min) and $^{14}_{7}N$ (γ, n) $^{13}_{7}N$ ($\tau = 10$ min), all induced by photons, can be used for the determination of oxygen, carbon and nitrogen. Photons of higher energy than those emitted by radioactive atoms must be employed. Some workers have used X-rays from a betatron[133]. The energy of the γ-radiation from mesothorium is sufficient, however, for the reaction $^{2}_{1}D$ (γ, n) $^{1}_{1}H$. Thus deuterium can be assayed through the neutrons produced, despite the fact that no radioactive substance is formed in the reaction of the deuterium itself[134, 135]. The neutrons are determined by one of the methods given in the next section.

In conclusion, we mention the procedures in which no radioelement is produced directly or indirectly, but 'instantaneous' nuclear reactions take place with the emission of charged particles. The quantity of the element is determined by measuring the (relative) number of nuclear reactions occurring per unit time during the bombardment. The most important example is the determination of boron; the isotope $^{10}_{5}B$, which occurs to the extent of 20% in natural boron, undergoes the reaction $^{10}_{5}B$ (n, α) $^{7}_{3}Li$ with slow neutrons. The reaction releases a large amount of energy, and the fast moving heavy particles, $^{4}_{2}He$ and $^{7}_{3}Li$, can be counted with an ionization chamber (p. 71) or with a photographic plate (p. 87)[136–138].

6. Absorption Analysis

In absorption analysis beams of slow neutrons are the most common form of radiation used. Slow neutrons are absorbed specifically by some elements, and therefore such elements can be determined through the decrease in the intensity of the beam. In contrast to activation analysis, the production of radioactive nuclides or the emission of heavy fragments is not essential. The limit of detection is never very low, however.

As they are uncharged particles of low energy, slow neutrons cannot be measured directly. Two main types of apparatus are used to measure the flux of slow neutrons: neutron counters and detectors. The sensitivity of the measurement naturally depends upon the energy of the neutrons, since the reaction cross sections depend on it.

The neutron counters generally make use of the instantaneous reaction ${}^{10}_{5}B$ (n, α) ${}^{7}_{3}Li$, the cross section of which is high. Such counters are called 'boron chambers'; the energetic He and Li nuclei produced in each event give rise to pulses of ionization, which are counted. Boron trifluoride counters are used most frequently; these are proportional counters filled with gaseous boron trifluoride, or, in some cases, the walls of the counters are coated with solid boron. The number of pulses is in any case a measure of the flux of slow neutrons, always assuming that the energy spectrum is constant.

Neutron detectors consist of elements which form β-active isotopes when slow neutrons are captured. The detector is introduced into the beam of neutrons, and the induced β-activity is measured later. This activity is proportional to the neutron flux if the energy distribution is constant. Detectors of indium foil or of manganese dioxide powder are often used. The radioelements produced (${}^{116}In$, ${}^{56}Mn$) have half-lives of 54 min and 155 min, respectively.

Standard samples whose chemical composition should be as similar as possible to that of the sample being analyzed are used for calibration. In other words, the determinations are always relative. Absolute measurements, using the numerical values of the absorption coefficients (reaction cross sections), are hardly practicable; the absorption spectra for the slow neutrons are too complicated.

The details of the application of absorption analysis to individual elements cannot be discussed here; we refer the reader to the review literature[1, 109, 139–142]. Among the elements which lend themselves best to assay by absorption analysis are Li, B, Cd, In, Hf, Ta, Mn, Ag, Au, Rh, and some of the rare earths.

A special case is that of hydrogen. Though not absorbing slow neutrons very much, hydrogen scatters them strongly; procedures for analyzing hydrogen can be based upon this fact. The (slight) hydrogen content of perfluorinated hydrocarbons has been determined by measuring the decrease in the intensity of a beam of thermal neutrons[143]. The measuring device must in this case subtend only a small solid angle, when viewed from the sample; otherwise the scattered neutrons may reach it. This rapid non-destructive procedure might well find applications in biochemistry.

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CHAPTER X

ABSORPTION AND EXCRETION OF ELEMENTS

1. Introduction

In this chapter we shall discuss the principles which govern the absorption and excretion of chemical elements by animals and plants—particularly the former. Unavoidably, this chapter is oriented physiologically as well as biochemically. It is of course impossible to catalog the many investigations which have been made in this field with radioelements; for this, we refer to compilations in the literature, most of which are arranged by elements[1–7].

A study of the uptake of radiolead (thorium B) by plants was published in 1923, and investigations of the distribution of the bismuth isotope, radium C, in living human beings followed in 1927 (p. 1). These studies were the forerunners of a vast number of investigations on the absorption, distribution, and excretion of elements. Such work is of primary importance, not only in general biochemistry and physiology, but also in nutrition, medicine, including pathology, toxicology, pharmacology and forensic medicine, and, finally, in agriculture.

The ability of isotopic methods to provide answers in this field is due both to the fact that they permit the detection of foreign elements in trace amounts and also to the fact that a stationary state can be maintained; the latter is important in the study of elements which occur naturally in the body. Information can therefore be obtained not only about the equilibrium distribution, but also about the velocity of transfer processes. In other words, both analytical and kinetic investigations of the metabolism of the elements are possible.

Balance experiments on the absorption and excretion of radioelements by animals and human beings are of course carried out *in vivo*. The distribution of radioelements among the various organs can also often be investigated *in vivo*, both when the elements remain in solution, and also when they accumulate as insoluble substances. Body fluids or tissues can be taken for the radiochemical analyses. In certain cases the radiation leaving the body can be measured directly[4, 8–13] (see p. 83). Even soft β-rays, like those from radiocarbon, can be measured as they emerge from the ear of a mouse[14]. ^{32}P and ^{90}Sr in human beings have been measured externally through their bremsstrahlung[15,16]. A particularly large number of *in vivo* studies have been devoted to the uptake of iodine by the thyroid[17–19]. In order to determine the content of radiophosphorus or radioiodine, the blood of a living animal was pumped continuously through a hole in a scintillator; discrimination was possible between the flashes of light produced by the two elements[20]. A review of the technique of biochemical (enzymological) work with living plants has been published[21].

It is nonetheless true that most work on the accumulation and distribution of radioelements is performed after the death of the subject. It is often important to stop the metabolic processes immediately after death. This can be done by lowering the temperature, or—selectively—by the addition of certain metabolic poisons. The clean separation of tissues from accompanying fluids is another problem. It is necessary in certain cases to standardize the experimental procedure, if reproducible results are to be obtained.

2. Membranes in Organisms

The distance which substances introduced into a living organism, or their metabolic products, can travel depends upon the permeability of the many 'membranes' present in the body. These membranes are extremely varied in nature. Besides the simple membranes, there are multicellular layers with complicated structures, like the mucous membranes of the stomach and intestines; the substances must in this case pass through a number of different individual membranes. Even the apparently simple membranes, like the cell-wall itself, sometimes prove to be multi-layered. Isotopic investigations have been made of the cell-walls of yeast (p. 128), and of erythrocytes (p. 131), for example. Finally, although they are difficult or impossible to demonstrate anatomically, the coatings of cell constituents (microsomes, for instance) act as membranes.

The structures of the membranes are also quite varied. Membranes are known which are more or less homogeneous in structure, whereas others exert their effects by virtue of their porosity. Formally, at least, all spatially defined obstacles to the movement of solvents and dissolved substances can be regarded as membranes. The physico-chemical theory of membranes in general, and the mechanism of action of individual membranes have been the subjects of thorough investigation[1, 22–35].

Since physiological membranes usually impede the motion of the solvent (water) and of the dissolved substances, including ions, to different extents, the membranes can be regarded as being fundamentally 'semipermeable'. In the limiting case, they are completely impermeable to certain substances. This limiting case occurs quite often in organisms, particularly with substances of high molecular weight. For instance, proteins do not pass at all through many kinds of membranes.

Passage through membranes proceeds by various mechanisms, depending upon the type of membrane and solute, and on other conditions. Water and water-soluble substances may diffuse through polar portions of membranes or pass through water-filled pores; filtration or ultrafiltration thus occur (sieve-action). Fat-soluble substances may be dissolved in the lipids on one side of a partition, diffuse through, and be released again on the other side. Fats can also be partially hydrolyzed and emulsified, and thus pass through pores[36].

The net transport is the difference between the rates of flow in the two directions. Without using isotopes, only the net transport can be measured with certainty. But when isotopes are employed, the flow in one direction can be determined by radioactivity measurements, so that the flow in the other direction can be calculated by comparing it with the net transport; or, conversely, the net transport can be calculated from the flow in both directions, by working with two different isotopes of the same element. An especially useful check consists in comparing the chemically-determined net transport with that calculated from the flow in both directions, obtained by radioactivity measurements. The flow in both directions can obviously be measured even when the net transport is zero or very small. Physiological equilibrium may be defined as a state of zero net transport. This state need not be based on equality of concentration (or thermodynamic activity, to be exact) between the two phases.

In many important cases, the concentration differences are surprisingly great. For example, some cells of the animal body, particularly muscle [37, 38], liver[39] and red blood cells (p. 131) contain much higher concentrations of potassium ions than the extracellular fluid, and yet the cell walls are permeable to potassium. Muscle may contain, per unit weight, 60 times as much potassium as the extracellular fluid[37, 38]. Yeast takes up potassium ions against a concentration gradient of 3000 : 1[40–43]. For phosphate, see[44–47].

As we have stated, an exchange of material occurs even at physiological equilibrium, where the concentrations on both sides of the membrane are no longer changing. As far as simple inorganic ions are concerned, it has been emphasized especially by Krogh[48] that the equilibrium state is based upon the continuous renewal of material. Apparently the 'dynamic state of body constituents', successfully investigated by Schoenheimer with regard to organic substances, also holds for the movements of simple ions.

Before isotopes were available, the permeability toward elements normally present in the body could be demonstrated only by showing that an increase of the concentration on one side of the membrane led to a net transport of material. However in this case unphysiological conditions prevail. The fundamental advantages afforded by maintaining stationary physiological conditions, as can be done with the aid of labeled atoms, will be discussed in more detail in Chapter XI.

The remarkable fact that different concentrations (more precisely, molar free energies) can exist on the two sides of a permeable membrane at equilibrium is explained by the use of the concept of 'active transport' [22, 24, 26, 27, 34, 35, 49–51]. Active transport is defined as any transport which cannot be explained on the basis of the physico-chemical properties of the phases which are separated by the membrane. Because of the complicated nature of the membrane it is found to be difficult to calculate what the 'passive' transport across the membrane would be; nevertheless, if the water does not itself show net flow, the simple relation

$$M_i/M_o = [c_o f_o / c_i f_i] \exp (zFE/RT)$$

holds for the ratio of the passive transport velocities into (M_i) and out of (M_o) the cell. The concentrations and activity coefficients of the ions in the two phases are represented by the terms c and f; z is the ionic charge, F the faraday, E the potential difference between the phases, R the gas constant, and T the absolute temperature [22, 24, 26, 27, 49, 52]. M_i and M_o can be determined individually, as has been noted, if two different isotopes of the same element are available. Otherwise M_i or M_o must be compared with the net transport ($M_i - M_o$). If the value of M_i/M_o differs from that calculated from the formula, active transport is assumed to occur.

Because of electrostatic effects, the ion of opposite charge also follows, if the membrane is permeable to it, even though it is not subject to active transport. Active transport has also been observed in the cases of unionized or slightly-ionized compounds, like amino acids and carbohydrates, and is indeed very important in these cases as well. The question naturally arises here whether the compounds are changed chemically during or after passage through the membrane [34, 35, 52–58].

Active transport, therefore, causes an acceleration or a retardation in the net transfer which would result from the properties of the two phases alone; in the extreme case, the net transfer is reversed. The net transfer ceases when the active transport is balanced by the reverse diffusion due to the increased concentration. The equilibrium has then been attained.

Since the active transport cannot be ascribed to the properties of the two homogeneous phases, it must be caused by processes occurring in or on the membranes. Because it differs from the 'natural' flow, it requires work, which comes from the consumption of free energy. Energy continues to be consumed even at equilibrium, if the molar free energies of the ion on the two sides of the membrane are different while the membrane remains permeable. Ussing, indeed, considers active transport as a transport resulting from energy furnished by the cell. Metabolic poisons, such as DNP (dinitrophenol), may inhibit active transport either partially or completely.

There is no doubt that active transport involves the participation of enzymes. The latter are specific and transport certain substances only. When ions are concerned, we may speak of an 'ion pump'. It is usually assumed that the transported substances are temporarily bound to carriers which ferry them across the membrane, although other mechanisms have been proposed[26, 50, 57, 60–62]. 'Exchange diffusion' is defined as the movement of a solute across a membrane in strict mole-for-mole exchange with a similar solute moving in the opposite direction[22, 63]. The presence of a carrier is of course insufficient to explain the active transport, since, as has been stated above, it is necessary to provide energy. It has been suggested that the energy is made available through energy-rich nucleotides[64].

A classical example of a membrane capable of active transport is the skin of the frog, which was investigated by DuBois-Reymond as early as 1857, and again by Krogh in 1937. The two velocities, M_i and M_o, for the transport of sodium ions have been measured individually by the simultaneous use of the two isotopes ^{22}Na and ^{24}Na. Over a certain range of concentrations, the ratio of these quantities was found to be 100 times greater than would have been calculated for passive transport. In the case of potassium (where only one radioisotope was available, and the flow in one direction had to be compared with the net flow) a smaller, or even negligible, effect was observed. The counter-ion, chloride (^{36}Cl and ^{38}Cl), is transported passively under the influence of the potential gradient due to the movement of the sodium[24, 25, 27, 59, 60, 62, 65, 66].

Experiments have also been performed with frog skins, in which equal concentrations of ions on both sides of the membrane were established by bathing with Ringer solution, and equal potentials, by applying a voltage. When the net flow, $M_i - M_o$, was determined for each ion, with radiosodium, -potassium, or -chloride, it was found to differ from zero only in the case of sodium. The transport of sodium ions corresponded exactly to the electric current measured in the circuit (Fig. 17).

The cell-wall of the erythrocyte has been examined particularly thor-

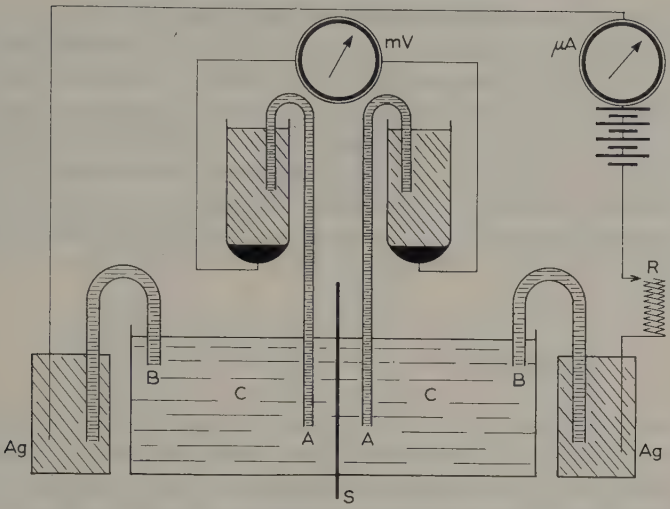

Fig. 17. The frog skin S separates the electrolyte solution C into two parts. The solution is well mixed and aerated. The potential across the skin is measured with the electrodes A, and can be made equal to zero by means of a flow of current B—B[24, 25, 62, 67].

oughly[68, 69]. Potassium is concentrated in the erythrocytes of most mammals[1, 28, 70–76]; however, the red blood cells of some species store sodium [77–79]. In any case, the labeled substances (here sodium and potassium) are exchanged through the cell wall[80]. The counter-ions, chloride[81] or bicarbonate[82], follow the alkali ions passively. Orthophosphate passes through the cell-wall in a complicated manner[57,83–87]. The behavior of other mammalian cells toward phosphate has also been investigated[85, 88–90].

Here the many important isotopic investigations of the role of alkali cations in muscle cells[22, 24, 25, 28, 49, 72, 73, 91–92a] and nerve cells[22, 24, 25, 49, 72, 73, 91–98] can only be mentioned. The mucous membrane of the stomach actively exchanges chloride ions for bicarbonate; the process has been followed quantitatively with ^{36}Cl, ^{38}Cl and ^{14}C. Sodium is passive in this system. The acidification of the stomach can in part be explained by this mechanism[22, 24, 25, 49, 91, 99–103].

Investigations of permeability by means of labeled atoms have of course also been made with water itself[22, 24, 25, 49, 91]. The skins of frogs and toads have been examined with heavy water[104, 105]. The permeability of turtle eggs[106], dog intestine[107] and of lower[108, 109] as well as higher plants [110, 111] has also been investigated with heavy water. The results are quite remarkable, but not easy to interpret[62].

If the substance passing through the membrane is subject to irreversible processes, like chemical alteration by enzymes, further transportation, or deposition in an insoluble form, a steady state may be reached, but not an equilibrium state. A constant, unidirectional flow of material then takes place. This flow of material through membranes of living organisms is in many cases quite pronounced. Such membranes, like the mucous membrane of the stomach, are therefore in an unphysiological condition when the flow is brought to a halt in an experiment *in vivo* or *in vitro*[99]. Transport and storage are the basis of the distribution of elements and compounds in the bodies of animals and plants.

3. The Administration of Elements to Animals

The results of experiments on absorption depend upon the time course of the dose and on the manner in which the radioelement is administered. In the case of the higher animals, for example, ingestion (oral administration), injection (intravenous or intramuscular), inhalation (as gas, droplets, or dust), or absorption through the skin may be used.

When substances are administered via the alimentary canal, they may be taken up by the walls of the stomach or intestine. Substances not absorbed from the digestive tract eventually appear in the feces. When they are absorbed, they are usually excreted in the urine if they are excreted at all, but they may also appear in the stool, by way of the bile. Finally, it is necessary to consider excretion through the skin, and, in the case of elements which are volatile or form volatile compounds, excretion by the lungs (exhalation).

Emanations which are produced at a constant rate from radium or thorium X deposited in the body constitute a special case. Since the emanations occur only in the form of free atoms, they are excreted as such from the body sooner or later, mostly in the expired air, although only if they have not already disintegrated; the relatively long-lived radon is more likely to be lost by excretion than is thoron. It has been demonstrated that shortly after the uptake of radium only a small fraction of the radon is eliminated, but that the fraction increases with time, and eventually—after years have passed—reaches 70%[112, 113].

An example of the effect of the method of administration on the manner of excretion is the following: under certain conditions, when the vitamin was given orally, radiocobalt from labeled vitamin B_{12} was excreted to the extent of 80% in the feces and only of 1% in the urine, but parenteral administration resulted in 50% excretion in the urine and only 6% in the feces[114]. Of course the distribution of the radioelement in the body also depends on the method of administration (p. 138, 146).

An example of a study of absorption through the intact skin is given by the absorption of P-labeled tricresyl phosphate; a large uptake of this dangerous poison was observed in man and in the dog[115]. Gases, *e.g.*, radon, also penetrate the intact skin[116]. *Amoeba proteus* absorbs labeled protein from solution by pinocytosis[117].

Techniques of administering radioactive substances to laboratory animals, and of taking samples from them, have been discussed in reviews [4,5,118]. In these and some other[1,6] reviews the results obtained by various procedures are surveyed.

4. The Administration of Elements to Plants

The administration of tracers to higher plants through the roots, through the leaves (infiltration), by injection, and by implantation has been described. The excretion of elements and compounds has been studied less thoroughly in the case of plants than in that of animals. Radiophotosynthesis is discussed in Chapter XI.

Because of its tremendous importance, administration through the roots has been much investigated by plant physiologists and agronomists. Here again the uptake of ions against a large concentration gradient is possible. Thus the absorption is not in every case due to simple (passive) diffusion, but may proceed through a non-random process, catalyzed by specific enzymes, and requiring the consumption of free energy[50,51,119–123a]. The hypothesis has been put forward that the 'outer space' of the roots can be entered by diffusion, either freely on subject to Donnan equilibria, and the ions are subsequently moved into the 'inner space' by active transport[51,123a]. Amino acids are concentrated in carrot slices against a concentration gradient[124].

Isotopic investigations of the uptake of fertilizers frequently serve the practical needs of agriculture[4,5,125,126]. The absorption of uranium fission products from the soil has also been examined[127,128]. (For strontium, see p. 135.) Simple organic compounds, *e.g.*, labeled dimethyl-acrylic acid [129], may also be taken up through the roots. It is noteworthy that the generally autotrophic higher plants are very well able to utilize sucrose administered by way of the roots[130]. Similar experiments have been performed with ^{35}S-methionine and ^{32}P-nucleic acids[131]. In Chapter XI we shall return to the uptake of CO_2 through the roots.

Low-molecular weight substances (salts, simple organic compounds) are taken up directly by leaves, as is proved by agriculturally-important studies on leaf-fertilization[132–143a]. (In the laboratory the leaf may simply be floated on the active solution.) On the other hand, the labeled substances can be washed out of the leaves by rain[140, 141, 143a–145]. The fission products ^{90}Sr and ^{137}Cs from bomb ash are also absorbed[146]. The penetration of labeled materials can be accelerated by vacuum-infiltration [21, 147–153]. A review on the permeability of plant cells is available[154].

Plants can be injected in various organs, like leaves or stems. A modification of the injection procedure consists in scratching or cutting the parts of the plant which are above ground, and introducing the labeled material into the wounds. A procedure, which is especially important in practice, is the simplest: the stem of a cut plant is placed in a vase of solution.

An implantation procedure has been used in the study of the biosynthesis of lignin (p. 303). Labeled coniferin, the incorporation of which into the new lignin was to be demonstrated, was implanted under the bark of a spruce sapling, the cut surface sealed with grafting wax, and the distribution of the radiocarbon in the sapling after a certain time was determined [155, 156].

After labeled substances have been taken up in the sap of higher plants, they are 'translocated' to other organs. The rate of translocation is often surprisingly high, and may vary from one substance to another under identical conditions. The factors responsible for the rates and for the changes in the concentrations during the flow are at present under investigation in many laboratories[50, 51, 121, 157–166].

A special problem, which illustrates the capabilities of the isotope method, arises in the case of the peanut. To what extent can the fruits, growing under the ground, derive their mineral constituents directly from the ground? It was shown with labeled atoms that calcium must be directly assimilated by the fruit, while sulfur can be brought in by way of the stem[167, 168].

5. The Materials Balance in the Animal Body

Qualitatively, some features of the uptake and excretion of materials by the animal body, *i.e.* of the 'balance' between input and output, may be anticipated. For simplicity let us assume that (long-lived) radioelements are present in chemically stable forms, *e.g.* as monoatomic ions or as anions of the oxygen-acids.

If the organism is to continue living, the continuous administration of a substance must finally lead to a stationary state. After this state has been reached, the administration, on the average, is balanced by an equally rapid excretion. Since this is true for every isotope of an element, the specific activity of the element excreted at equilibrium must be equal to that of the element administered.

Let us first compare the uptake with the excretion, neglecting the radioactivity. By chemical analysis one finds out, whether or not the stationary state has been attained. If the output of the element is equal to the uptake over a fairly long period of time, the organism is probably in equilibrium with respect to this element. We shall not discuss in detail the certainty of this in any given case, in view of the analytical errors and the fluctuations in the metabolic activity of the individual.

In the case of 'physiological' elements (those which occur naturally in the body) the equilibrium is usually attained quickly. It is always established rapidly, for instance, when chloride ions are administered orally or intravenously to mammals, which do not store this element. In the cases of iodine and calcium ions, which are accumulated—in the thyroid and the bone, respectively—the equilibrium is also reached quickly; the body cannot be forced to take up much more than the natural amount of an element for which it possesses an excretion mechanism.

This is not necessarily true of those elements which do not occur naturally in the body. The possibility of attaining equilibrium is then determined by whether or not the chemical properties of the element cause it to be stored in the organism. For example, the foreign element bromine comes to equilibrium rapidly, because it distributes itself essentially only in the extracellular space (p. 141). On the other hand, hardly any kind of equilibrium can be attained with the alkaline earth elements radium, barium, or strontium. These elements do, to be sure, behave much like calcium and are therefore stored in the bones, where they replace some of the calcium[169–178] (see also p. 136); however, since they are poisonous, and since therefore the ratio of the amount of calcium in the bone to that of the foreign element hardly ever corresponds to an equilibrium value, the excretion of these elements cannot in practice equal the uptake.

Such an 'unlimited' accumulation does not occur only when the body confuses a foreign element (Ra) with a physiological one (Ca). Experiments have shown that orally administered plutonium (in the form of plutonium or plutonyl ions) is partially deposited as a colloidal hydroxide in the bone marrow and liver, without replacing any naturally occuring element. The same thing can happen in the cases of physiological elements if they are administered in forms which can not be assimilated by the body, as, for example, phosphorus as colloidal chromium phosphate (see below).

Even in the case of unlimited accumulation, only a part (usually a small part) of the element administered is taken up permanently, the remainder being excreted either immediately or in the further course of metabolism. For example, radium injected intravenously is taken up permanently (by the bones) to the extent of only about 1%, despite the fact that an equilibrium condition, where a large fraction of the calcium would be replaced by radium, cannot be even remotely approached.

Calculations and experiments may also be made to investigate the extent to which equilibrium can be obtained with respect to the (long-lived) radioisotope content of the element excreted, and hence, its specific activity. If we consider the elements mentioned above, it is obvious that the unphysiological elements, bromine, plutonium and radium, will be excreted at constant specific activity from the very beginning. In the case of the naturally-occurring element, potassium, constant specific activity must be reached very quickly, because the potassium in the body is present in a readily-exchangeable form. Similar conditions are obtained on administration of iodine in the form of iodide, despite the fact that the element is stored in the form of an organic compound.

On the other hand, in the cases of calcium and other elements which occur in the body in large quantities, and only partly in exchangeable form, constant specific activity of the excreted element cannot usually be obtained; the specific activity of the calcium excreted increases continuously, but the calcium of the bone is never completely exchanged[1, 4, 5, 170, 175, 179–190]. It is true that even in this case a constant activity of the eliminated element can be forced, and an equilibrium thus simulated, if such large amounts of the labeled element are introduced that only a minute fraction can be taken up by metabolic processes.

If the radioelements do not have sufficiently long half-lives, the specific activity of the excreted material must, because of radioactive decay inside the body, be less than that of the material taken up. This is true, for example, of ^{42}K ($\tau = 12.4$ h). By measuring the decrease in the specific activity it is—with the approximately correct assumption of complete exchange—possible to determine the average time the element remains in the body, and hence the amount of element present in the stationary state.

When an organism has attained a stationary state in respect to an inactive element, the subsequent oral administration of the labeled element permits one to answer the important question, to what extent the element passes unchanged through the digestive tract, or, on the contrary, is absorbed, but re-excreted at the same rate. The contributions of the two mechanisms depend upon the experimental conditions, such as the size of the daily dose and the physiological condition of the individual.

As an example, labeled phosphate fed to mammals is mostly absorbed, and re-excreted later. The phosphorus excreted immediately after the start of the experiment is therefore only slightly active; the phosphorus excreted is, as we say, largely 'endogenous'[1, 89, 191, 192]. Calcium (see above) behaves similarly. In an interesting series of experiments, female mice were treated before and during pregnancy with radiocalcium, in order to obtain uniformly-labeled offspring. The length of time during which the total calcium was retained by the next generations could then be determined[185].

In contrast, a mammal saturated with iron can hardly absorb any more (labeled) iron from an oral dose[1, 193–197]. It therefore excretes iron with unchanged specific activity from the very beginning ('exogenous iron'). The iron administered is not taken up by the iron-storing protein of the intestinal mucosa, apoferritin[198–200]. In iron deficiencies, however, orally administered labeled iron appears rapidly in the hemoglobin[1, 194]. The iron metabolism of the fetus has also been studied[201]. By using both isotopes ^{55}Fe and ^{59}Fe, it is possible to study simultaneously the uptake of intestinal iron by the plasma and the transfer to other tissues of plasma iron bound to protein[202]. Electronic calculators have been used to solve the complicated mathematical problems in the kinetics of iron metabolism[203].

In the study of nutrition, and in stockbreeding, it is important to distinguish between endogenous and exogenous elements in the feces[5, 180, 204–207]. Some definitions and formulas applying to the uptake and excretion of radioelements are given in Section *9*.

6. Storage in Organs

The previous considerations have been applied, for the most part, to the entire body of the animal. Detailed investigations of the distribution of the elements in various organs naturally provide more information; such studies involve both chemical determinations and measurements of activity. It is thus established which organs take up certain elements selectively. Many such investigations of the storage and distribution of radioelements have been made.

The distribution of the element in the body—administered in the same chemical form—may depend to a large extent upon the dose[171, 208]. (It is possible to vary the dose while keeping the total activity constant, either by dilution with an inactive isotope, or by going from a more active radioisotope to a less active one (*e.g.*, ^{90}Y to ^{91}Y or ^{224}Ra (Th X) to ^{226}Ra). This fact can also be expressed by saying that some organs have a large affinity, but only a small capacity, for certain elements, whereas other organs initially do not absorb the element avidly but in the long run have more space to absorb it.

After the administration of a small dose, therefore, the element appears primarily in organ A, and after a larger dose, in organ B. If radioelements of equal specific activities are used in both cases, most of the total activity is found in A, in the first case, but in B, in the second case. The failure to consider this fundamental fact, or to consider it sufficiently, has robbed many a biochemical study of its value!

In selecting the dose to be used in a study, the natural content of the element in the body must be considered. It would be pointless, for example, to use carrier-free radiosodium for studies on man, since there is so much inactive sodium in the body. On the other hand, when radioyttrium (which is foreign to the body) is introduced, it is a matter of great importance whether the radioyttrium is carrier-free, poor in carrier, or diluted with a large quantity of inactive yttrium.

Carrier-free yttrium injected intravenously is a 'bone-seeker'[209]; in contrast to the alkaline earths, it is deposited in the bone matrix[210]. On the other hand, if large quantities of inactive yttrium are added, the radioyttrium is stored in the liver, kidneys, and spleen[182, 211, 212]. The distribution of the radioyttrium depends also upon the method of its administration[213]. Obviously a knowledge of these matters is of great importance in therapy with radioisotopes.

Iodine provides a particularly simple and important example of the way in which the absorption may vary with the dose. Carrier-free iodine is taken up entirely by the thyroid in a very short time. The total normal content of iodine in the human thyroid is only about 7 mg, however. This figure cannot be much exceeded. Therefore, when radioiodine is diluted with even a few milligrams of inactive iodine, merely a small portion can be taken up by the thyroid, and the rest is soon excreted[17, 187, 214–220] (see p. 62).

When 1 mg of carrier silver was added to radiosilver (corresponding in this case to an isotopic dilution by a factor of 6×10^7), the uptake of radiosilver in the spleen of a rat after intramuscular injection was increased by a factor of 300, and the uptake in the liver, by a factor of 100[208, 221, 222]. Toxicological studies with the very poisonous element, beryllium, showed that isotopic dilution by a factor of 10^6 led to a greatly increased uptake by the spleen and liver of rats injected intravenously, but to a diminution of uptake by the bones[222, 223].

7. 'Spaces' (Body Compartments)

Because of the selectivity of the membranes in the body of an animal, only certain parts of the body fluids are accessible to solutes introduced into the blood stream. These parts are known as body compartments, or 'spaces', and can vary for different solutes.

First, however, we shall discuss the experiments performed with the solvent itself, *i.e.*, with water. These experiments were carried out with heavy water, D_2O [224–228], and with super-heavy water, T_2O [228–230]. A small quantity of water with a known content of the heavy or radioactive isotope was injected intravenously, then small samples of blood were drawn at certain time intervals, and analyzed for the isotope. If v_1 and v_2 are the volumes of the water injected and of the water compartment (the volume accessible to the injected water), and A_1 and A_2, the isotope contents (in the case of tritium, the specific activity also) of the water injected and of the sample, then the relation

$$A_1/A_2 = v_2/v_1$$

must hold. Because of the formal similarity of this expression to that given on p. 111, the expression 'isotope dilution method' is used here as well.

In actual experiments, account must be taken of the loss of water by excretion of urine, by exhalation, and by perspiration. The error so introduced is not large, however, because the circulating water attains constant isotope content so rapidly. The injected water is almost completely at equilibrium with the extracellular water within a few minutes, and with the total water in one hour [227–229, 231–233]. (When the water is given orally, somewhat longer periods are required.) The space available to injected water in man usually ranges from 50–65% of the body volume; it depends upon sex, age, the state of health, the fat content, and other circumstances [227, 234, 235].

In general, no account need be taken of the rapid exchange of hydrogen between water and acids, alcohols, sugars, and amines. Although the loss of isotopic hydrogen makes the water compartment appear too large, a quantitative estimate indicates that the error thus committed is insignificant (1–2%) [225, 226, 235]. However, if the experiments were protracted (in which case, of course, it would be necessary to allow for the uptake and excretion of hydrogen-containing compounds, including water), a more pronounced decrease in the specific activity of the circulating water would be observed, since in due course all the hydrogen of the body is gradually replaced in metabolic processes.

Attempts to measure the sizes of the body compartments by means of dissolved substances were made even before isotopes came into use. Dilution experiments were carried out, not with isotopes of substances normally present in the body, but with substances foreign to the body. With the aid of dyes, for instance, the plasma volume was found to be 5% of the body weight [236–238]. The extracellular space as a whole was determined with sodium thiocyanate [239, 240], sodium thiosulfate [241, 242] or carbohydrates [243, 244] and was found to represent about 20% of the body weight. The intracellular space is much larger than the extracellular space; the total body water was measured with antipyrine[244, 245].

Because radioactivity is so easily measured, even substances foreign to the body have lately been employed in radioactive form. The volume of the plasma has been determined by the use of suspended particles of chromium ^{32}P-phosphate[246].

In principle, however, it is preferable to use labeled substances which are normal body constituents. It is only in this way that physiological conditions can be maintained, so that the interpretation of the results is not subject to criticism. Obviously each substance has a definite ability to pass through the various membranes of the body. Therefore the volume available to a given substance is characteristic only of that substance, and the identification of this volume with one of the idealized spaces conceived by the physiologist is dubious. Of course, isotopic labeling is necessary with naturally-occuring substances. The stationary state concentrations are maintained if tracer quantities only (of high specific activities) are employed[1, 235, 244].

The total body water of man has been determined with ^{15}N-urea[247], that of cats, with ^{14}C-urea[248]. Radioactive sulfate is recommended[244] for determining the extracellular space. The plasma volume is measured with serum albumin labeled with radiobromine or radioiodine[250–252]. Strictly speaking, these substances do not occur naturally; it would be better to use serum proteins with labeled carbon or sulfur (see p. 230). The space accessible to the erythrocytes is related to the plasma volume, but not identical with it; this volume can be determined by the use of erythrocytes labeled in the iron or phosphorus[253, 254]. The change in the blood volume and in the number of circulating blood cells resulting from loss of blood (shock) has been investigated with erythrocytes using two labels (^{32}P, ^{51}Cr); the differently-labeled erythrocytes were injected at different times[255] (*cf.* also p. 38).

Sodium is at first confined to the extracellular fluid, but slowly penetrates into the cells and especially into the mineral of the bone (see below). Consequently, the sodium space is very dependent on the time of measurement, so that a complete definition of this volume must include a statement of the time interval between injection and measurement.

It has already been emphasized that cells can selectively accumulate certain ions, even though the ions do not undergo chemical reactions inside the cells. In other words, the ions are neither converted to other chemical compounds nor deposited as precipitates. A good example is potassium, which accumulates in many types of cells. Hence the potassium space is larger than would be expected on the basis of the geometrical volume available to the ion. 98% of the total potassium is intra-

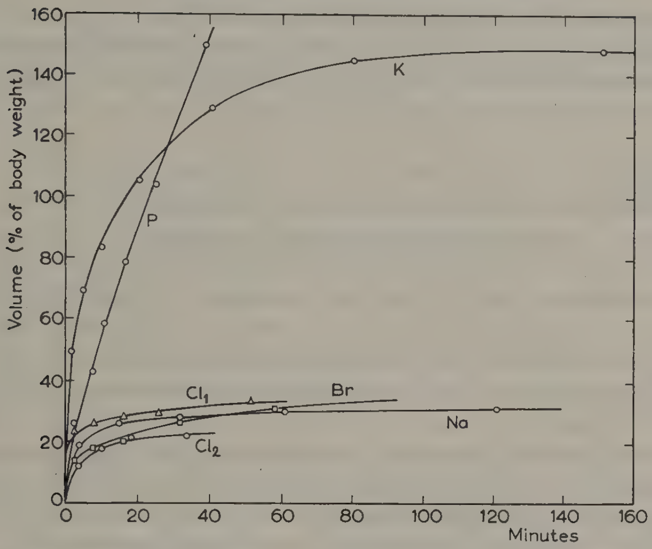

Fig. 18. Time course of the dilution of various labeled ions in the plasma of the rabbit (L. Hahn and G. Hevesy[1]).

cellular; a small part of the sodium, on the other hand, is found inside cells[1, 76, 227, 235].

Some characteristic curves obtained in the determination of body spaces with simple ions are presented in Fig. 18. It will be observed that sodium (measured over a short period), chloride, and bromide give rather similar results.

In the determination of the 'spaces' of the body by use of solutes, the total quantities of these substances are obtained as a sort of by-product. Again, it must be remembered that the exchange requires time, and that not all of the element is necessarily present in exchangeable form[256].

The quantities of exchangeable potassium[257] and sodium[227] in the normal human body have been found to be 48 and 43 milli-equivalents/kg, respectively; the female body, which usually contains more fat, has less potassium[70, 76]. However, whereas potassium exchanges fairly quickly[1, 76, 258], half of the sodium contained in the bone – corresponding to 20–25% of the total body sodium – is almost non-exchangeable[1, 76, 259–263].

The exchangeable quantity of organic compounds can also be determined in some cases. We have already noted that the body water can be measured with urea. The urea in the mammalian body was determined by measuring the amount as well as the isotope content of the urea in the body fluids. It was found by the use of labeled uric acid that the exchangeable amount of this substance is much larger in patients with gout than in normal human beings[264–267]. The exchangeable cholesterol of rats has also been measured[268].

8. The Effect of the Chemical Form of the Element and of the Physiological State of the Organism

Except in the last section, we have not considered the possibility that an element may be introduced in different chemical forms. Nor have we taken account of the fact that the element or the compound may undergo chemical transformations within the organism. The consideration of these problems is actually very important.

The case of sulfur[268a] has often been examined, and it will therefore be used as an example. Radiosulfur has been used, for instance, in the forms of sulfate, sulfide, thiocyanate, free sulfur and sulfur-containing amino acids[1, 2, 269–271]. Sulfate accumulates in small quantities in bones and cartilage[272–278]; it is also used for the synthesis of amino acids and of taurine in ruminants[279–281] and chickens[282, 283]. Ruminants can even assimilate elementary sulfur. Sulfide is partly incorporated into amino acids, and partly oxidized to sulfate and excreted. Accordingly, the distribution of sulfur in the organism much depends on the chemical form in which it is presented. In conclusion we merely mention that radioactive sulfur has been administered in the form of synthetic drugs[269, 270], mustard gas[270], cysteamin[284], biosynthetic penicillin[270, 285–288] or plasma protein[270] (p. 231). Characteristic results have been obtained in each case. The distribution of radiosulfur from CS_2 has been studied in toxicological investigations[249, 289, 290]. Plants are capable[291] of utilizing the sulfur of sulfate[4, 292] and elementary sulfur[293].

Beryllium labeled with ^{7}Be migrates preferentially to the bone and remains there when the citrate complex is administered, whereas colloidal beryllium (or ionic beryllium, which quickly becomes colloidal) is stored in the liver, and is excreted again more quickly[222, 294, 295]. Similar situations exist with gallium[296, 297], yttrium[212] (p. 138) and chromium[298]. Detailed discussions of the effects of complex formation are to be found in the literature[171, 299].

The metabolism of labeled halogens in organic halogen compounds, like tetraiodofluorescein[300] or bromotriphenylethylene[301], is different from that of simple halogen ions. The halogen of the latter, but not of the former, organic compound has been observed to separate from the organic compound.

An extreme example of the influence of the chemical state of the element is provided by phosphorus[1, 2, 88, 89, 302] (p. 136). If radiophosphorus in the form of orthophosphate is injected, it is actively removed from the blood by the cells. It then undergoes many chemical transformations in the body, and is incorporated into the bones, so that very high values for the size of the phosphate space are obtained, although no definite value can be reached. On the other hand, the insoluble compound, chromium ^{32}P-phosphate, is after intravenous injection stored almost entirely in the liver and spleen where it remains indefinitely. No appreciable uptake of chromium phosphate by other organs occurs[303, 304] (p. 140).

Many other examples of the influence of the chemical state on the distribution of a radioelement in the body can be found in investigations using carbon-labeled organic compounds; these will be discussed in detail in the following chapters. At this point, we merely refer to a toxicological study made on monkeys with carbon-labeled CCl_4[305].

The physiological state of the organism has a great bearing on absorption. The situation is relatively well-defined in the case of calcium uptake by bone, which is impeded by a lack of vitamin D[3, 186, 306–310]; calcium excretion is enhanced by a number of unrelated agents[311]. The distribution of radiobromine in the nervous system of cats and dogs depends upon the functional state[312]. The absorption of radiocobalt, in vitamin B_{12}, is different in healthy subjects and pernicious anemia patients, and is influenced by the administration of intrinsic factor[313] (see also p. 132).

9. The Excretion of Radioelements by Animals

After the administration of a radioelement has been discontinued, the time course of the excretion by the whole organism or by an organ depends upon the nature of the radioelement, the manner of its administration, the previous history of the system, and the conditions during the excretion. We shall assume temporarily that the radioelement has a long half-life.

In the simplest case, the amount of radioelement excreted per unit time ($-\mathrm{d}A/\mathrm{d}t$) is proportional to the quantity present (A). The rate then corresponds formally to that of a first-order reaction. We have

$$-\frac{\mathrm{d}A}{\mathrm{d}t} = \mu A, \text{ or } A = A_0\, e^{-\mu t}$$

An exponential excretion curve, completely analogous to the decay curve of a radioactive substance, is obtained; only, instead of the decay constant $\lambda = 1/\tau_m$, we have an excretion constant, $\mu = 1/\theta_m$. τ_m is the average life of the radioactive element, θ_m the average time the substance remains in the organism. The half-life τ, as is well-known, differs from the average life τ_m, by a factor $\ln 2 = 0.692$. In an analogous fashion, we may define a biological half-life $\theta = \theta_m \ln 2$.

In the stationary state, where the chemical composition of the organ remains constant, the biological mean life of the isotope corresponds to the chemical turnover time of the element concerned; the isotope excreted is completely replaced by the inactive element. The excretion constant can then be considered as a turnover rate, or, what comes to the same thing, as a renewal rate. The rate as thus defined has the dimension time^{-1} and is the reciprocal of the renewal time of the 'pool' (p. 158)—that is, of the total quantity of the substance available for the process under consideration[314, 315]. The quantity of the element in the pool need not be known. Some biochemists, however, define the turnover or renewal rate as the amount of substance which reacts per unit time[316–318]. The latter quantity, according to the proposal of one author[319], can be better called the 'flux rate' of the element. It is unfortunate that different definitions are employed side by side.

All these quantities can in the stationary state be measured just as well by determining the uptake as by measuring the excretion of isotope. It makes no difference at all whether radioactive atoms are replaced by inactive atoms, or vice versa.

A study of the concept of turnover reveals many errors which may arise as a result of inaccuracies in the definitions, of a lack of knowledge of pathways, or of the attempt to base the calculations on too few experimental points[320].

The exponential nature of the curve proves that atoms are 'chosen' for excretion in a random fashion, and not on the basis of the length of time they have been present in the organ. For example, the decrease (disappearance clearance) of radiosodium injected into the human gastrocnemius muscle followed an exponential curve; 4–5% of the quantity present was lost every minute because of local circulation[98, 321]. A half-life of 28 days over a period of several months has been reported for the removal of uranium from human bones[322]. (Other authors find longer half-lives). Isotope experiments show that hydrogen is initially excreted by man with a biological half-life of 10 days[225, 235]; in rats the half-life is 3.3 days, and in mice, 1.1 days[323]. In all cases, there remains a residue which is excreted more slowly[324]. The loss of gas from tissues has been studied with radioactive inert gases (A, Kr, Xe), and conclusions have been drawn regarding the speed of gas exchange between lungs and other tissues (*cf.*, p. 132)[325].

The radioactive half-live of the element may not be long enough for the radioactive decay during the period of excretion to be neglected. This applies, for example, to ^{24}Na ($\tau = 15.0$ h). We have

$$-\frac{dA}{dt} = (\mu + \lambda)A, \text{ and hence}$$

$$A = A_o e^{-(\mu+\lambda)t} \text{ and}$$

$$A = A_o \exp\left(-\frac{\theta_m + \tau_m}{\theta_m \tau_m} t\right)$$

The half-life, as influenced both by disintegration and by excretion, may by called the effective half-life,

$$\delta = \frac{\ln 2}{\mu + \lambda} = \frac{\ln 2}{1/\theta_m + 1/\tau_m} = \frac{\theta_m \tau_m}{\theta_m + \tau_m} \ln 2$$

Of course, $A = A_o 2^{-t/\delta}$. A value of 11.5 h has been quoted[326] for the effective half-life of ^{24}Na in man (see also[327, 328]).

However, not nearly all activities in the body decrease at rates which are even approximately exponential. Deviations from the exponential curve lead to the conclusion that the radioelement is present in the organism in various states of combination. The concept of the state is to be interpreted in its broadest sense; besides the nature of the chemical bond we must also consider the distribution in various parts of the organism (or of the organ). Deviations from exponential decay occur in the case of foreign as well as of physiological elements[329].

One example is the excretion of radium from the human body, though no differences in chemical binding are expected in this case. In the first period after the uptake of the element the excretion is relatively rapid; in other words, the ratio of the amount excreted per day to the amount still present is high. Later, this ratio declines steadily. Presumably at first the portion of the radium is eliminated which is located at easily accessible points, *i.e.* at the surface of the bones. The larger the portion of retained radium which is located in the 'inner', more inaccessible parts of the skeleton, the slower is the excretion (*cf.* p. 135). It was found empirically that the excretion follows not an exponential law, but a power law [112, 113]. One form of such a law, which held for a group of human subjects under certain conditions, was

$$A/A_o = 0.54\, t^{-0.52}$$

where A/A_o is the fraction still retained at time t (expressed in days).

In other cases, the excretion could not be represented by a power of the time, but—again purely empirically—by a sum of exponentials. The retention of radiocalcium in the blood of a calf after a single intravenous injection followed the equation

$$A = A_o(0.42e^{-2.1t} + 0.32e^{-0.38t} + 0.022e^{-0.07t} + 0.037e^{-0.0039t})$$

(t in minutes)[5]. The chemical bonds of the calcium, like those of the radium, must have been uniform.

Cats[330] retained the fraction

$$A/A_o = 0.284e^{-0.243t} + 0.415e^{-0.0234t} + 0.302e^{-0.00119t}$$

of radiocarbon injected intravenously as bicarbonate (*cf.* p. 203). An analogous experiment was carried out on rats[331]. The carbon of organic substances is, of course, excreted at extremely varied rates, but in any case much more slowly than that of bicarbonate. Similar considerations apply to hydrogen[324]. The time course of the expiration of radiocarbon will be discussed in section XII, *4*. Excretion formulas in the form of exponentials have also been given for radioiodine under various physiological conditions[325, 332, 333].

The manner of administration of the element may make a great difference even though the chemical state of the element may not be subject to changes. It has been reported, for example, that plutonium—which is predominantly tetravalent in organic media—has a biological half-life of 10 years when ingested, but of only two months when inhaled. In the former case the plutonium is stored mainly in the bones, and in the latter case, in the liver (see also p. 136). No such difference has been found in the case of polonium (also tetravalent) whose effective half-life is always 82 days; the half-life of radioactive decay is 137 days[326].

The investigations of Hevesy and of Schoenheimer, which have rightly been considered classics, have shown that most parts of living organisms are subject to renewal, and hence, to the loss of radioelements taken up in physiological form. When all is said and done, the 'exogenous' element iron differs from the 'endogenous' elements calcium and phosphorus only quantitatively. Nonetheless, the average biological life of some elements may exceed the lifetime of the individual[194]. It is possible to apply the concept of the biological life to chemical compounds, or even more complicated entities, *e.g.*, erythrocytes[1]. It it is then necessary, of course, to consider the possibility that various parts of these units are renewed at different rates (see Section XI, *1*).

10. Artificial Methods for Increasing the Excretion of Radioelements

The biological, and hence the effective, half-life of a radioelement can in some cases be decreased by chemical treatment. This is important in the decontamination of human beings who have accumulated a dangerous quantity of radioactive material, *e.g.*, by accident[187].

The first idea which comes to mind is that of isotopically displacing the traces of the radioactive element by overloading with inactive isotopes of the same element. Experience proves, unfortunately, that this method is seldom effective. Radiocalcium is displaced only very slowly by inactive calcium. Vitamin B_{12} with labeled cobalt cannot be 'washed out' with an excess of the inactive vitamin[334]. Evidently the vitamin in the body is not readily accessible for exchange (*cf.* p. 141).

When inactive isotopes are not available, displacement by similar elements, or their compounds, may be tried. For example, calcium competes with radium, and zirconium with plutonium. The deposition of plutonium can indeed be reduced by the administration of zirconium; the mechanism of the displacement is complicated, since both elements have a tendency to form colloidal solutions[335].

Treatment with complexing agents is often effective[171, 299, 336–338]. It is possible, for example, to enhance the excretion of the fission product radioyttrium by injecting citric acid in the form of zirconium citrate[339]. The excretion of radioyttrium and of plutonium can be accelerated greatly with the strong complexing agents EDTA (ethylenediaminetetraacetic acid), and diethylenetriaminepentaacetic acid even after deposition in the bones[182, 340–343]. The excretion of calcium[344] and lead[345] is also improved. For the removal of plutonium, the simultaneous use of zirconium citrate and of complexon is particularly recommended; the two complexing agents are said to have a complementary action, involving both bone and soft tissue[346]. A pessimistic view is taken of the possibilities for the removal of radiostrontium with chelating agents[347].

Methods of removing radioactive elements, *e.g.* calcium and strontium, from circulating blood by passing it through an ion-exchange column or through an artificial kidney have been studied. Values for the maximum rate of mobilization of the alkaline earths from bone can be derived from such experiments[348].

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CHAPTER XI

THE PRINCIPAL PROBLEMS OF INTERMEDIARY METABOLISM

A. PHOSPHORYLATIONS AND PHOTOSYNTHESIS

1. Introduction

The investigation of intermediary metabolism probably constitutes the most important biochemical application of labeled atoms. The fundamental principles are the same here as those upon which the experimental and theoretical studies of the uptake and excretion of the elements are based, and which were treated in Chapter X. Accordingly, the definitions and formulas given there may also be applied to the problems of intermediary metabolism. However, whereas the chemical reactions of most other elements are limited in scope, and hence have only limited physiological significance, the manifold reactions of the compounds of carbon constitute the very heart of life.

In this chapter we shall begin by showing, with appropriate examples, how the isotope method can be used to elucidate the details of intermediary metabolism. In Chapter VIII we have already described the radiochemical analytical procedures employed in the analysis of metabolic intermediates and products. At that point, however, we developed a sort of static picture of the tissue at one particular moment, while in the present chapter we shall stress dynamic considerations, and follow the pathways of the reactions[1].

The synthesis (*e.g.* photosynthesis) and the degradation (*e.g.* glycolysis) of organic substances involve long sequences of reactions. It is important, therefore, to learn the order in which the reactions occur. The general problem is to discover all the steps between the reactants and the products, or, in other words, to find the precursors of all the organic compounds which appear. The problems of the reaction sequences are intimately connected with those of the reaction mechanism and of the enzymes involved. Finally, a question of great significance is that of the reversibility of biochemical reactions, such as phosphorylation. All these problems can be investigated with isotopes.

The reaction velocities can also be studied. The investigation of the uptake and excretion of inorganic elements has shown that in living matter they are often in a 'dynamic state'. We may anticipate that a similar dynamic condition is often observed in intermediary metabolism; most organic compounds, though they may seem only to be stored, are in a state of constant flux. Not only the location, but also the chemical state of the elements is in dynamic equilibrium; *i.e.*, synthesis and degradation are constantly taking place[2, 3].

The rate of turnover and the renewal time (p. 144) in the stationary state can be determined only with the aid of isotopes. Different parts of organic molecules may be renewed at different rates. Thus in the case of the phosphatides, as Hevesy has emphasized, it is necessary to distinguish between the turnover of the base, of the fatty acid, and of the phosphoric acid. In the case of nucleic acids, the turnover times of the bases, the sugars, and the phosphoric acid are all different. In short, when a turnover time is given, it must be made clear to what entity it refers. Quite generally, kinetic investigations are significant only to the extent that the reaction mechanism is understood[4].

The organic substances of the body are present in metabolic pools of greater or smaller sizes, which are enlarged by fresh synthesis, and diminished as their material is consumed. The metabolic pool of an organism (or of an organ, or even of a cell) in respect to a particular substance has been defined as that mixture of compounds (derived from ingested nutrients or produced by degradation) which the organism (organ, cell) uses for the synthesis of the substance[5]. The pool of a given amino acid, for example, consists of all of the amino acid, which is present as such or in the form of an easily-utilized precursor, to the extent that it is *immediately* available for synthesis. The word 'immediately' indicates that the pool cannot be defined strictly. For example, amino acids are released by relatively slow degradation of proteins, and then also become available. Every pool is therefore more or less inhomogeneous[4, 6]. Systems of equations have been given for calculating the sizes of such pools[5].

In Section III, *1* we referred to the chemical exchanges of atoms and groups of atoms between molecules, and of molecules between different phases; these exchanges can be demonstrated with isotopes. An exchange, for example, of carbon dioxide between the gas and the carboxyl group of an acid may be regarded as a case of chemical exchange, even though it requires the presence of a biogenic catalyst (enzyme). The observation of the exchange of isotopic atoms or groups has recently been employed as a useful indicator of enzymatic action, for instance in phosphorylation, as will be shown in the next section.

Before isotopes were available, enzymes had to be demonstrated in systems which were not in the stationary state. The presence of the enzyme was proved by showing that the addition of more reactants led to the formation of more products. When isotopes are employed, however, and the transfer of the isotope in either of the two directions of the reaction is demonstrated, the stationary state can be maintained. Moreover, an important practical advantage is the fact that it suffices to isolate some of the labeled substance, produced in the reaction, in the pure state from the system; no quantitative determination is necessary.

The existence of a dynamic state of organic body constituents, which will be discussed in concrete detail in subsequent chapters, is demonstrated by experiments which from a formal point of view resemble experiments on chemical exchange. A labeled substrate is added to the biological system under investigation; after a time another substance is isolated from the system, and the concentration of the labeled atoms in this substance is measured. However, in contrast to simple chemical exchange, the dynamic state is the result of the overlap of a number of distinct processes. Usually some of these processes can be classed as synthetic, and others as degradative; if the system is in a stationary state and does not change its overall chemical composition during the time of the experiment, the degradative processes must just be compensated by the synthetic processes.

This compensation requires multi-angular processes (reaction cycles) where the forward reaction proceeds by a path different from that of the back reaction. For example, glycolytic synthesis (if the term is permissible) follows a pathway which differs in part from that of glycolytic degradation (p. 189). Accordingly, the individual reactions through which radiocarbon of lactic acid is incorporated into glycogen in the reversal of glycolysis, are not at all stages the same as those in the splitting of glycogen to lactic acid.

The fundamental difference between an exchanging chemical system (for example, $KI + \overset{*}{I}$) and a dynamic (living) system consists in the fact that the chemical exchange may proceed even when the system is, with respect to its chemical composition, but without considering differences between isotopes, in a condition of minimum content of free energy. This is not true for a system in a dynamic state, *e.g.* a system where glycogen and lactic acid coexist. If the one-sided conversion of glycogen to lactic acid, which would proceed rapidly in the presence of the characteristic enzymes, is to be compensated, free energy must be supplied from outside. Very often, this free energy is stored and transported in 'energy-rich' bonds. The need to consume free energy in the stationary state may be the most important characteristic of living matter[333].

We turn now to the clarification of biological reaction mechanisms. To demonstrate the reaction path A → B, the reactant A is introduced with a label, and the product B is then isolated ('trapped'). Whether or not B is further converted to C, can be shown by adding B in labeled form and measuring the activity of C. This procedure is of course applicable even when the concentration of B in the stationary state is small.

It is also possible to add, together with the labeled A, unlabeled B (a 'bank') and to demonstrate a decrease in the activity of C as a result of the dilution of the radioactive B. Thus the radiosynthesis of B, which may be more difficult than that of A, is avoided. In this way, the utilization of organic sulfur compounds for the biosynthesis of penicillin was studied with labeled sulfate only[7, 8]. It was also shown by this procedure that proteins of Ehrlich ascites tumor incorporate isoleucine, but not alloleucine[9].

Without labeled atoms, the reaction pathway A → B → C can be demonstrated only by showing that the administration of increased quantities of A (B) leads to higher concentrations of B (C). But this would be unphysiological. Moreover, nothing would be seen if the reaction velocity were limited by the amount of enzyme present. Such a procedure, for instance, would hardly suffice to show the formation of sex hormones, which occur in very small quantities, from cholesterol, which is already initially present in large amounts.

In the case of heterotrophic microörganisms which thrive best on one particular organic substrate, but can use another nutrient (such as glucose) in the absence of the preferred substrate, the preference for the specific substrate can be demonstrated by offering it in the unlabeled form, and noting the resulting inhibition in the formation of labeled metabolites from the labeled unspecific nutrient. Thus the necessity of adding a series of labeled substrates ('isotopic competition method'[10–13]) is avoided.

In kinetic studies, the speeds of turnover of A, B and C are measured. While under the present assumptions A can only decrease in amount, and C can only increase, a stationary state may exist in respect to B. The turnover of B in the stationary state can be determined from the speed with which labeled molecules (derived from A) enter the pool of B, or—if B, but not A, is labeled—from the speed with which labeled molecules leave the pool. In the stationary state, both rates must be equal; if the reaction sequence A → B → C is the only possible one, all the velocities, which can be measured with labeled atoms, must be equal.

In actual fact, however, we often observe parallel reaction paths, leading from the same reactant to the same product; in other cases the reaction paths are branched. Which pathway predominates, may depend on conditions[14]. In order to elucidate these complicated systems, it is often necessary to use selectively-labeled or doubly-labeled reactants, or to employ simultaneously several substances labeled with different radioelements.

A powerful method for studying complicated mechanisms consists in the determination of the order in which various labeled reaction products appear after the introduction of a labeled starting material. If the reactions are rapid, the samples must be taken at short intervals. The incorporation of labeled phosphorus into organic phosphate esters by yeast was studied at intervals of fractions of a second[15]. Short-term experiments were also required in the search for the primary products of photosynthesis (p. 168).

In many cases it is not enough to know the reaction sequence qualitatively; in order to understand the various reaction paths—which can of course occur simultaneously—it is necessary to carry out a mathematical analysis on the basis of the time course of the specific activities of the compounds which appear. The required set of equations depends on the particular characteristics of the system[16,17] (*cf.* also p. 158).

When a stationary state is compared with a non-stationary state, the application of labeled atoms makes it possible to decide whether the transition is due to an acceleration of the forward reaction, or to a slowing of the back reaction. For example, the question can be answered whether the change from a resting condition to a state of growth is brought about by increased synthesis[18,19] or by decreased degradation[20] of proteins, since the rates of synthesis and of degradation can be measured separately.

For the isolation of labeled metabolic products it is often necessary to add weighable amounts of 'carriers' (p. 18). Here the possibility must be taken into account that the carrier may not reach equilibrium with the pool[21]. This may be the case if the spatial distributions of the labeled and unlabeled (carrier) substances are not identical. Such inequality in distribution may be caused by slowness in the diffusion and in the exchange of molecules[22]. In particular, membranes may delay the establishmentof equilibrium[23].

The interfering membranes can, under some circumstances, be destroyed by suitable measures. For example, in the investigation of the respiration of yeast they were ruptured by freezing[24,25]. An interesting way of eliminating the influence of membranes without drastic treatment has been employed in the study of *Aerobacter*. In order to determine the utilization of labeled citrate by this microörganism, strains grown on acetate and on citrate were compared. Since the permeability of the cells is probably identical in both cases, the observation that only the latter strain utilized labeled citrate was taken as proof that the strain grown on acetate does not produce citrate and metabolize it further[26–28].

2. Phosphorylation

It is well known that phosphorylation reactions, where the phosphorus is converted from inorganic phosphate to an organic phosphate ester, or transferred from one organic molecule to another, occupy a central position in the biological synthesis and degradation of organic compounds. These reactions can be studied with the aid of radiophosphorus or radiocarbon; many results have been obtained since the first investigations in this field[29–31]. A wealth of material regarding these problems has been presented in two symposia[32].

Labeled esters are often required for the experimental study of phosphorylation. The study of possible methods of syntheses of such esters often sheds light on the (enzymatic) mechanisms of the phosphorylations concerned. At the second of the symposia mentioned above, Greenberg reported the procedures which are useful for the analytical separation of the labeled phosphorus compounds[33,34]; a practical survey is also found in Aronoff's book[1].

Compounds can be labeled by esterification with radioactive phosphoric acid, or by enzymatic reaction with ^{32}P-ATP. The notation used here (analogous to writing ^{14}C-glucose) does not indicate anything about the position of the radioactive phosphorus atom or atoms. (The position of the radiophosphorus is, unfortunately, only seldom known; analytical studies, in which the activities of the individual phosphorus atoms of ADP or ATP are determined separately, shed light on the mechanisms of the reactions by which the nucleotides are obtained, and at the same time open the way to the biosynthetic[35–37] or abiosynthetic[38] preparation of selectively labeled nucleotides (see p. 37).) In this section ^{32}P will denote labeled orthophosphate ion, and ^{32}PP, pyrophosphate, unavoidably labeled in both phosphorus atoms. P and PP are the corresponding unlabeled ions.

For example, labeled hexose monophosphate has been obtained enzymatically from glycogen and phosphate ion, and labeled hexose diphosphate from hexosemonophosphate and radioactive ATP[29,30]. ^{32}P-ADP can be made from ^{32}P-ATP by usual preparative methods[39]. In one case, adenylic acid, radioactive orthophosphate, and the oxidizable substrate β-hydroxybutyric acid were incubated with the proper enzymes to produce ^{32}P-ATP. Thereafter, the terminal phosphorus atom was removed by treatment with glucose and hexokinase[40]. It has been shown that in phosphoribosyl pyrophosphate (PRPP; see below) each of the two pyrophosphate phosphorus atoms is derived from a specific phosphorus atom of ATP after reaction of the latter with ribose-5-phosphate[41].

Labeling can proceed even after equilibrium has been established, if labeled phosphorus is added in a suitable chemical form. It is necessary, of course, that the reaction velocities from left to right and from right to left (which must be identical at equilibrium) are sufficiently great. This procedure may be regarded formally as labeling by exchange, although no direct transfer of phosphorus atoms or phosphate groups occurs between the radioactive compound introduced and the compound to be labeled (*cf.* p. 158). For example, radiophosphorus introduced in the form of ATP into the system

$$\text{phosphopyruvate} + \text{ADP} = \text{pyruvate} + \text{ATP}$$

is converted to phosphopyruvate even when the system is in chemical equilibrium[42]. Similarly, the glucose phosphate becomes labeled[43] when ^{14}C-glucose is introduced into the equilibrium:

$$\text{glucose} + \text{ATP} = \text{glucose-6-phosphate} + \text{ADP}$$

Hexose phosphate (and hexose diphosphate) can presumably also be labeled by phosphate exchange under conditions of chemical equilibrium (see above).

Hexokinase is an example of a transferase; it transfers orthophosphate groups. Other transferases transfer nucleotide residues or phosphoribosyl groups to suitable substrates, and at the same time release free orthophosphate. Examples are:

$\text{NMN} + \text{ATP} \rightleftharpoons \text{DPN} + \text{PP}$ (NMN = nicotinamide mononucleotide)[44,45]
$\text{adenine} + \text{PRPP} \rightleftharpoons \text{AMP} + \text{PP}$ (PRPP = phosphoribosyl pyrophosphate; p. 258)

Reactions of these kinds in some cases also make it possible to label compounds by exchange at equilibrium. Later it will be shown, using the formation of acetyl coenzyme A as an example, that it can be concluded from the existence of such an exchange reaction that a sequence of reactions includes a partial reaction of this type.

As far as the position of the phosphorus is concerned, the sequence of reactions may involve a removal of orthophosphate[40, 46–51] or of pyrophosphate[52–57] from ATP, *viz.*

$$\text{ATP} = \text{ADP} + \text{P} \quad \text{or} \quad \text{ATP} = \text{AMP} + \text{PP}$$

This applies for example, to the formation of acetyl coenzyme A (see p. 165). The specific substrates of the enzymes, therefore, do not appear explicitly. Although the radiophosphorus distributes itself between the participants of an over-all reaction of this type, it is obviously impossible to deduce the nature of the substrate from this fact alone. In some cases, the nucleotides of other bases replace those of adenine[58–60]. Literature reviews give many more examples of such exchange reactions of phosphorus-containing groups[61, 62].

Labeled atoms have recently become one of the most important tools for elucidating the mechanisms of catalysis by enzymes. We shall first present a few examples of phosphorylating reactions which take place without the participation of inorganic phosphorus. Afterwards, we shall refer to reactions where phosphate or pyrophosphate is produced or consumed.

One would not expect phosphate to exchange during enzymatic reactions in which the position of the phosphate in the molecule is not altered. Experiment confirms this prediction; no label enters the ester when glyceraldehyde-3-phosphate is oxidized to 3-phosphoglyceric acid in the presence of $^{32}PO_4$ ions[31].

Even when the phosphate group changes its position in the organic molecule, it may still not be able to exchange with free phosphate ions. For example, the intramolecular isomerization of glycerophosphate ($\beta \rightarrow \alpha$ isomer), which is catalyzed by acid, does not labilize the phosphorus sufficiently for exchange with inorganic phosphate[63]. The same is true for the isomerization of glucose-1-phosphate to glucose-6-phosphate by phosphoglucomutase—an intermolecular reaction; no exchange with radioactive inorganic phosphate ions takes place here[31]. Neither do the glucose esters become radioactive during isomerization in the presence of carbon-labeled glucose[64].

On the other hand, the radiophosphorus and radiocarbon originally present in one of the three compounds, glucose-1-phosphate, glucose-6-phosphate, or glucose-1,6-diphosphate (the coenzyme of phosphoglucomutase), distribute themselves uniformly among all three compounds when the equilibrium is established, as would be expected[65]. In an analogous manner, ^{32}P is uniformly incorporated into 2-phosphoglyceric acid, 3-phosphoglyceric acid, and 2,3-diphosphoglyceric acid (the coenzyme) when labeled phosphoglyceric acid is isomerized enzymatically[66]. These questions are discussed in a general review of enzymatic isomerization[67].

With respect to intermolecular isomerizations, Hevesy[68] has come to the conclusion 'In all these cases, a direct transfer of phosphate groups from one molecule to another took place without passing through the inorganic stage. A possible explanation of the above results is that, for example, phosphoglyceric acid and adenylic acid form a molecular complex with the enzyme, the phosphate radical is shifted from one molecule to the other, and then the products, phosphoglyceric acid and adenosine triphosphate, leave the enzyme surface. The labeled inorganic phosphate present in the solution then clearly has no opportunity to participate in the reactions.'

Frequently exchange reactions can occur only when all the substances, which appear in the chemical equation for the enzymatic reaction, are present at the outset, or can be formed. For example, to obtain labeled ADP by the hexokinase reaction (p. 162) it is necessary to have not only ^{32}P-ATP and enzyme, but also the substrate (glucose and/or glucose-6-phosphate); enzyme, ^{32}P-ATP and inactive ADP are insufficient because no turnover can occur with them alone.

Labeling does sometimes occur in the absence of certain reactants, however; this is explained by resolving the overall reaction into partial reactions. We illustrate this situation with an example which has been thoroughly investigated. A phosphorylase from *Pseudomonas saccharophila* (p. 34) synthesizes sucrose from glucose-1-phosphate and fructose. It was found that this enzyme catalyzes the exchange of phosphorus between glucose-1-phosphate and inorganic phosphate even in the absence of fructose or sucrose[69–71]. It was postulated, therefore, that a reversible reaction

$$\text{glucose-1-phosphate} + \text{enzyme} \rightleftharpoons \text{enzyme-glucose} + \text{phosphate ion}$$

occurred first. In contrast to this result, the phosphate ion was found not to exchange with glucose-1-phosphate in the presence of phosphorylase from muscle or potatoes. Obviously different mechanisms of enzyme action are involved[72]. Even in the case of the *Pseudomonas* enzyme, some authors suspect that traces of a glucosyl acceptor may be adsorbed on the enzyme; if this were true, the experimental basis of the mechanism proposed above would be undermined[62].

The formation of acetyl-CoA from acetate and coenzyme A is brought about by purified yeast enzymes and ATP. It has been found that phosphorus exchanges between pyrophosphate and ATP in the presence of enzyme and acetate alone, while the exchange of carbon between AMP and ATP requires acetate and CoA, and that of carbon between acetate and acetyl-CoA requires AMP and pyrophosphate. From these and other findings, the mechanism was concluded to be:

$$\begin{array}{rcl} \text{ATP} + \text{acetate} & \rightleftharpoons & \text{adenyl acetate} + \text{PP} \\ \text{adenyl acetate} + \text{CoA} & \rightleftharpoons & \text{acetyl-CoA} + \text{AMP} \\ \hline \text{ATP} + \text{acetate} + \text{CoA} & \rightleftharpoons & \text{acetyl-CoA} + \text{AMP} + \text{PP} \end{array}$$

Adenyl acetate is the mixed anhydride of adenylic acid and acetic acid. It will be observed that the enzyme does not occur explicitly in either of the two equations[73–77] (see also p. 193).

Many exchange experiments have been performed in recent years with the aim of demonstrating the existence of enzyme-substrate complexes analogous to that of the sucrose phosphorylase from *Pseudomonas*. The enzymes investigated include—besides phosphorylases—phosphatases, nucleotidases, dehydrogenases, etc. A list of these experiments, some of which yielded positive, and some, negative results, may be found in the literature, together with a general discussion of the ways in which labeled atoms can be used to elucidate enzymatic reaction mechanisms [78, 79]. Another review covers the study of enzyme reactions (mostly not phosphorylations) with labeled hydrogen [80, 81]. Radiophosphorus and heavy oxygen have been used to investigate the participation of ATP in muscle contraction [82–84].

The hydrolysis of glucose-1-phosphate has also been studied with heavy oxygen. Acid splits the C—O bond, but 'acid' and 'alkaline' phosphatases cleave the P—O bond. Muscle phosphorylase and sucrose phosphorylase split the C—O bond, no matter in which direction the reaction proceeds. This similarity in the behavior of the latter two enzymes is the more noteworthy since their mechanisms are quite different in other respects, as has been explained above [85] (see also [78, 86]).

Radioactivity can also be used to demonstrate the stoichiometric relationships in phosphorylation reactions. For example, in the reaction

$$\text{glutamylcysteine} + \text{glycine} + \text{ATP} \rightleftharpoons \text{glutathione} + \text{ADP} + \text{P}$$

the consumption of labeled glycine was found to be equal to the production of inorganic phosphate; therefore either one can be measured by determining the other [87, 88]. (See p. 239 for further data on glutathione.) The position of phosphorylation equilibria can be calculated from the radioactivity of one of the reaction products, even though it may be formed in very small amounts [31]. This procedure has been used to determine the free energy of hydrolysis of ATP [89–92].

Measurements of the ratio of the speed of incorporation of radioactive inorganic phosphate into ATP to the consumption of oxygen were used in an interesting manner to obtain the P/O ratio of respiring tissue suspensions in a stationary state. The P/O ratio is defined as the ratio of the number of equivalents of organic phosphate produced to the number of oxygen atoms consumed. It reflects the efficiency of the respiratory process (oxidation of substrate by elementary oxygen) or even the efficiency of individual steps of the oxidative chain (transport of two electrons via DPN and cytochrome C) [86, 93–96]. It is necessary to make sure that the ATP does not become radioactive by an enzymatic exchange with orthophosphate, independently of the oxidation of the substrate [97, 98]. Similar experiments were done with mitochondrial subfragments [99, 100]. Heavy oxygen has also been used to investigate the mechanism of oxidative phosphorylation in animal tissues [97, 101]. Other measurements have been carried out on plant mitochondria [102].

The literature contains some discussions of experiments, often difficult to interpret, on phosphorylation in intact animals [103–108]. Other work was concerned mainly with the differences between healthy and sick tissues, or those under unphysiological conditions; muscle[109, 110] and nerve[111, 112] tissues were discussed. Photosynthetic phosphorylation in plant tissues will be dealt with in the following sections.

Finally, the rates of phosphorylating reactions can be inferred from the transfer of radioactive atoms from 'left to right' or vice versa. At equilibrium, such determinations can be made only by isotopic methods. Under certain conditions *in vitro*, for example, the half-time for the renewal of the phosphorus in ATP was less than one minute in the oxidation of triosephosphate to phosphoglycerate; *in vivo*, the time amounted to only a few seconds[31]. The rate of the above-mentioned hexokinase reaction at equilibrium, as measured by the transfer af radioglucose, runs parallel to the concentration of the ADP or ATP, and drops to zero in the absence of, at least, catalytic amounts of these substances[43]. An attempt has been reported to determine the mechanism of action of 5′-nucleotidase by measuring reaction rates with labeled atoms[78].

3. Photosynthesis

The full potentialities of the isotopic method are revealed in the investigation of photosynthesis by green plants. It may indeed be asserted that only by the use of labeled atoms have we been able to obtain a more or less detailed understanding of the mechanism of photosynthetic assimilation. The hope is well founded that by the further use of this method every single step up to the synthesis of the simple carbohydrates will be elucidated within the foreseeable future. Progress in this field is chiefly due to Calvin and his school; preliminary studies were made by Kamen and Ruben[113–120]. Several reviews have appeared recently[121–130]. One review is devoted to the subsequent transformations of the sugars and other relatively simple substances in the plant[131].

The theoretical ideas and the experiments of R. Hill, Van Niel and others made it seem probable quite some time ago that the light energy absorbed was used to decompose water rather than carbon dioxide[132–135]. Under physiological conditions, the hydrogen thus made available for reductions undergoes a series of chemical transformations before it becomes active as reduced pyridine nucleotide—probably mostly TPNH[130, 136]—toward the assimilated carbon. The decomposition of the water has been experimentally divorced from the reduction of the carbon dioxide. It is still uncertain whether the chlorophyll plays any role beyond that of a sensitizer for the primary photochemical reaction[134]. In opposition to the majority opinion, O. Warburg—whose own work does not usually involve the use of labeled atoms—assumes that the carbon dioxide is directly bound to the chlorophyll, where it is reduced by the light[137–139].

It has been shown with heavy oxygen that the oxygen released arises from the water and not from the CO_2[140–144]. The isotopic composition of the oxygen in the sugar of the leaves, on the other hand, corresponds to that of the carbon dioxide[145]. Since the carbon dioxide is not directly reduced by the light, a limited assimilation of carbon dioxide occurs even in the dark; we shall discuss this phenomenon in more detail later.

The fundamental problems of the nature of the primary product of the assimilation of carbon and of its subsequent transformations have been solved to a great extent by the use of radiocarbon, particularly ^{14}C. We have already noted (p. 50) that isotope effects do not interfere with these experiments to an appreciable extent.

The following experimental technique has often been used. Suitable green plants, or their surviving organs, are 'fed' in the light with radiocarbon in the form of carbon dioxide or of dissolved bicarbonate ion, which is in equilibrium with carbon dioxide. The plants are killed, the substances formed are isolated by chemical methods, and examined individually for radioactivity. The activity of each substance indicates the amount of radiocarbon incorporated into it during the experiment[146]. A glass vessel suitable for the assimilation of radioactive carbon has been described by Hassid[1, 147].

The experiments are usually carried out under constant illumination. In this case the inactive CO_2 must be replaced by radioactive CO_2 at a given moment. Apart from the isotope content of their constituents, the plants are in a stationary state. In other cases it is better to reverse the procedure, keeping an atmosphere of radioactive CO_2 throughout, and suddenly turning the light on and off.

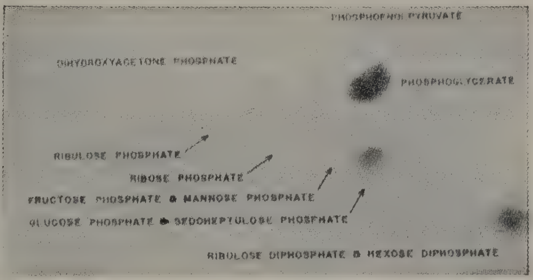
The longer the radiophotosynthesis—simultaneous action of $^{14}CO_2$ and light—is allowed to run, and the more time therefore the plant is given to transform the primary products, the larger the number of labeled compounds which can be detected by activity measurements. Conversely, to the extent that only a single 'first' product is formed from the radioactive carbon dioxide, a decrease in the time of radiophotosynthesis will finally restrict radioactivity to this one compound alone.

For these kinetic experiments, the single-celled algae *Chlorella* or *Scenedesmus* are frequently chosen. *Chlorella*, which can be cultured easily and reproducibly, was first used by O. Warburg 40 years ago to study photosynthesis. In the short-term experiments, a solution of radioactive bicarbonate is injected into the illuminated suspension of algae flowing through a glass tube, and thereafter the suspension is run into boiling alcohol. The hot alcohol immediately brings all enzymatic reactions to a standstill. Consequently, the radiocarbon can be metabolized by the algae for only a very short—and adjustable—time.

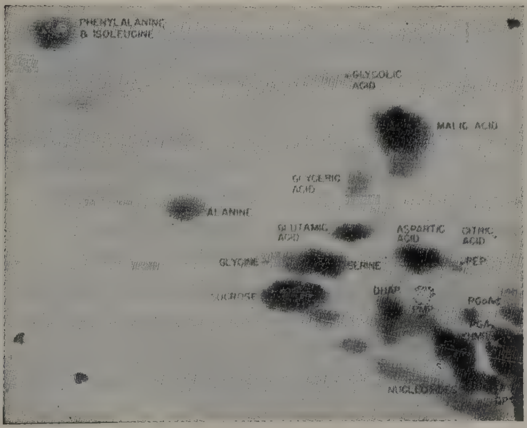
The analysis is performed by two-dimensional paper chromatography of the compounds originally dissolved in the alcohol. The amounts of the radioactive substances formed during the brief assimilation of radiocarbon are of course much smaller than those of the inactive, chemically identical materials originally present, so that the specific activities of these substances are low. However, they are sufficient. Qualitative identification is usually made by autoradiography, the paper chromatogram being pressed against a photographic film (Fig. 19); for quantitative measurements of the spots or the extracts of the spots the Geiger counter is preferable.

As the period of assimilation of radiocarbon was decreased—finally to only a fraction of a second—almost all of the labeled compounds seen on longer exposure disappeared. Only a single compound, 3-phosphoglyceric acid, retained appreciable radioactivity (see p. 173, however). Phosphoglyceric acid is therefore considered as the primary product of photosynthesis

Fig. 19. Autoradiograph of the paper chromatogram of an extract from algae (*Scenedesmus*), after assimilation of $^{14}CO_2$ in the light[119].


PHOSPHOENOL PYRUVATE
DIHYDROXYACETONE PHOSPHATE
PHOSPHOGLYCERATE
RIBULOSE PHOSPHATE
RIBOSE PHOSPHATE
FRUCTOSE PHOSPHATE & MANNOSE PHOSPHATE
GLUCOSE PHOSPHATE & SEDOHEPTULOSE PHOSPHATE
RIBULOSE DIPHOSPHATE & HEXOSE DIPHOSPHATE

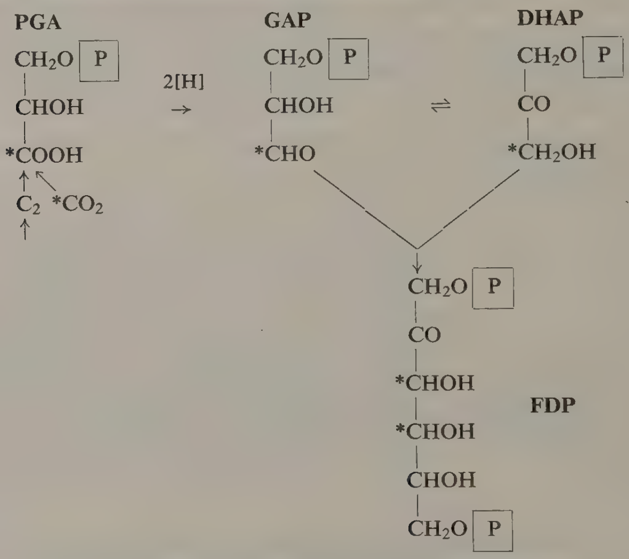
a) After exposure for 10 seconds. (Some locations are indicated, in which secondary products of photosynthesis are expected, but cannot be demonstrated).


PHENYLALANINE
& ISOLEUCINE
GLYCOLIC
ACID
MALIC ACID
GLYCERIC
ACID
ALANINE
GLUTAMIC
ACID
ASPARTIC
ACID
CITRIC
ACID
GLYCINE
SERINE
PEP
SUCROSE
DHAP
PGA
NUCLEOTIDES

b) After exposure for 10 minutes,

by *Chlorella.* Probably the same compound is also the primary product of other plants, and indeed the first steps in the synthesis of organic matter may be identical for all green plants, and thus all plants may be considered to operate, at least qualitatively, according to a uniform basic scheme [148, 149].

On the other hand, the fraction of the carbon stored in the form of carbohydrates, or used for the synthesis of fats, proteins, etc., may depend very much on the type of plant and on the physiological conditions—including the intensity and wave length of the light. The extent to which the newly-assimilated carbon is converted from the primary and the earliest secondary products to various successor substances can also be determined very nicely with radioactive tracers [150–160].



Formation of hexose diphosphate during photosynthesis.
P stands for the phosphate group.

For studying the reactions of phosphoglyceric acid (PGA), it proved useful to follow the sequence in which labeled carbon appears in various compounds. However, a bewildering assortment of labeled compounds are now formed in rapid succession. These compounds may arise by branching of the reaction sequences. In order to investigate the mechanisms it is therefore necessary not only to determine quantitatively the total activity of each compound as a function of the time, but also to establish the distribution of the radiocarbon inside the molecule at each moment. The stepwise degradation of the compounds and subsequent determination of the activities of the individual carbon atoms (p. 40) are therefore indispensable for the elucidation of the secondary reactions as explained, *e.g.*, in the text by Bassham and Calvin[161].

When the PGA produced in a short time was degraded, activity was found first in the carboxyl carbon. Phosphorylated glucose and fructose, with carbon atoms 3 and 4 labeled, also appeared within the first minute. It is thought, therefore, that phosphorylated hexoses (probably initially fructose diphosphate, FDP) are formed by condensation of two three-carbon fragments. On the basis of our knowledge of glycolysis (p. 189), it is presumed that the three-carbon compounds are glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP). A difficulty still lies in the fact that the distribution of the radiocarbon within the molecule of the hexose is not symmetrical[162, 163].

Calvin's work casts light on the reduction of PGA. Algae were first illuminated for a relatively long time in the presence of $^{14}CO_2$, and thus brought to a stationary state with respect to radiocarbon. The quantities, activities, and specific activities of all the simple products of both light and dark reactions thus became constant. The quantities of the primary and simple secondary products could therefore be determined by measuring their activities. The light was then suddenly turned off. Further analyses now showed that the quantity of PGA (as measured by its radioactivity) increased rapidly with time. It is obvious that in the stationary state this substance is consumed by a reaction for which the light constitutes the ultimate source of energy.

As has been said, the light reaction consists in the production of reducing power; this probably effects the conversion of phosphoglyceric acid to aldehyde. In glycolysis this enzymatic reduction is brought about by DPNH with the aid of energy provided by ATP. Suspensions of chloroplasts actually do reduce phosphopyridine nucleotide when they are illuminated, and liberate molecular oxygen in the process[164, 165]. In the presence of heavy water, deuterium is incorporated into DPN and TPN under the action of light[166]. In the light, tritium is transferred from water to chlorophyll, and from chlorophyll to TPNH[167].

On the other hand, it has been proved possible to reduce carbon dioxide to carbohydrates with cell-free plant extracts provided DPNH, ATP, and catalytic amounts of ribose-5-phosphate had been added[168, 169].

ATP is also synthesized by isolated chloroplasts, without the combustion of organic substances, such as members of the citric acid cycle, or the consumption of molecular oxygen; the occurrence of 'photosynthetic phosphorylation' can be recognized, for instance, by the fact that the radiophosphorus of phosphate ions is incorporated into ATP[130, 136, 170–180, 334]. In order to investigate photosynthetic phosphorylation, the technique of illuminating or depriving of light a plant material which was previously in a stationary state, has again proved useful; algae treated with radioactive phosphate can be used[181].

According to these results, it should be possible to carry out the whole photosynthetic process in cell-free systems. It was demonstrated quite early that plant-breis[182, 183], and partially-destroyed single cells[184] had a slight photosynthetic activity. Clear effects were finally obtained with suspensions of chloroplasts, and even with broken chloroplasts, or with fractions of chloroplasts[178, 185–192]. An integrated discussion of these results, from the standpoint of energy relationships, has been published[193]. For the biochemistry of isolated chloroplasts, see the reviews[194–197A].

The experiments of Calvin also led to the identification of the first carbon acceptor, that is, of the reaction partner of the carbon dioxide in its assimilation. The understanding acquired in this way made it superfluous to search for a 2-carbon compound which could act as an acceptor and form phosphoglyceric acid. Despite an intensive search, such a 2-carbon compound had never been found.

The work of Calvin showed specifically that the quantity of ribulose-1,5-diphosphate in the plant decreased rapidly after the end of illumination. Thus removal of the light blocked the synthesis of this compound, but not its consumption. On the other hand, when the stationary state was interrupted by suddenly removing the $^{14}CO_2$ while the light was kept on, the amount of labeled ribulose diphosphate rose, but that of phosphoglyceric acid decreased. Obviously, the ribulose diphosphate forms phosphoglyceric acid with CO_2 even in the dark, while the replacement of ribulose diphosphate from phosphoglyceric acid requires light. (Recently it has been suggested that the 1,5-diphosphate of the lactone of 2-carboxy-3-ketopentitol is an unstable first product of the reaction of ribulose diphosphate with CO_2[199].) On the basis of these and other experiments, Calvin has proposed a cyclic reaction scheme in which the ribulose diphosphate serves as the immediate acceptor of the carbon of the CO_2, and as a near precursor of the phosphoglyceric acid, while a fairly large number of steps must occur on the way from PGA to ribulose diphosphate. Not only the trioses and hexoses, but a tetrose, the pentoses, ribulose and ribose, and the heptose, sedoheptulose (or, to be precise, their phosphates) have places in the scheme[175, 200]. It is noteworthy that most of the sugars named, and of the enzymes involved, also appear in the oxidative degradation of carbohydrates; however, most of the reactions run in the opposite direction (p. 191).

Every completed cycle results in the assimilation of three molecules of CO_2 and in the carboxylation of three molecules of ribulose diphosphate. The six molecules of phosphoglyceric acid formed are reduced to six molecules of triose phosphate. Five of these are reconverted to ribulose diphosphate, while one molecule is transformed, via hexose phosphate, into sucrose or starch. This sixth represents the net increase in organic carbon. All of the enzymatic partial reactions, including that of ribulose diphosphate with labeled CO_2[201–203], have also been observed *in vitro*. Fig. 20 gives the ideas of Calvin's school with regard to the photosynthetic cycle.

The proposed mechanism explains how the radiocarbon finds its way into all positions of the members of the cycle, so that these compounds become labeled in all their carbon atoms. This is also true of the hexoses [206, 207]. Although some of the hexoses leave the cycle and form stores of carbohydrates, the hexoses and the reserve substances all participate in further metabolism; that is, a dynamic state prevails.

On the basis of further experimental results (an abrupt increase in the amounts of labeled citric and glutamic acid after the end of the illumination), Calvin has proposed a mechanism by which the phosphoglyceric acid formed in the light can be made immediately available to the citric acid cycle; this reaction sequence, which leads to pyruvic acid, takes place only after the illumination is cut off. Thus the PGA need not be converted to carbohydrate and degraded again to pyruvate. The inhibition of this reaction by light has also been investigated by other authors[208, 209].

In this connection, it is important to inquire to what extent the respiration of plants is altered by illumination. By the use of heavy oxygen, respiration can be followed independently of any simultaneous photosynthesis. Surprisingly large differences between different kinds of plants have been observed[210–216]. Heavy oxygen has recently been determined in photosynthetic experiments by activation analysis (p. 120).

The assimilation of carbon in the dark by green plants has been known for a long time, and constitutes strong proof that the appearance of reducing power and the assimilation of carbon are two separate phenomena. The products of the dark reaction are qualitatively identical with the products of assimilation in the light[215, 217–221].

A complicating factor is the possibility that carbon may not only be assimilated to form phosphoglyceric acid, but may also be bound in other chemical forms, *e.g.*, by β-carboxylation[130, 222]. Analogous processes are known to occur in microörganisms which do not exhibit photosynthesis, and also in animal tissues; they will be described in more detail later. In the absence of light, these side reactions usually become more prominent. Malic acid, for example, rapidly becomes radioactive[183, 223, 224].

Even the subterranean roots of the plants, despite their lack of chlorophyll, are able to bind and utilize carbon dioxide, as has been demonstrated quite clearly with radiocarbon[225–233]. In this case as well, the

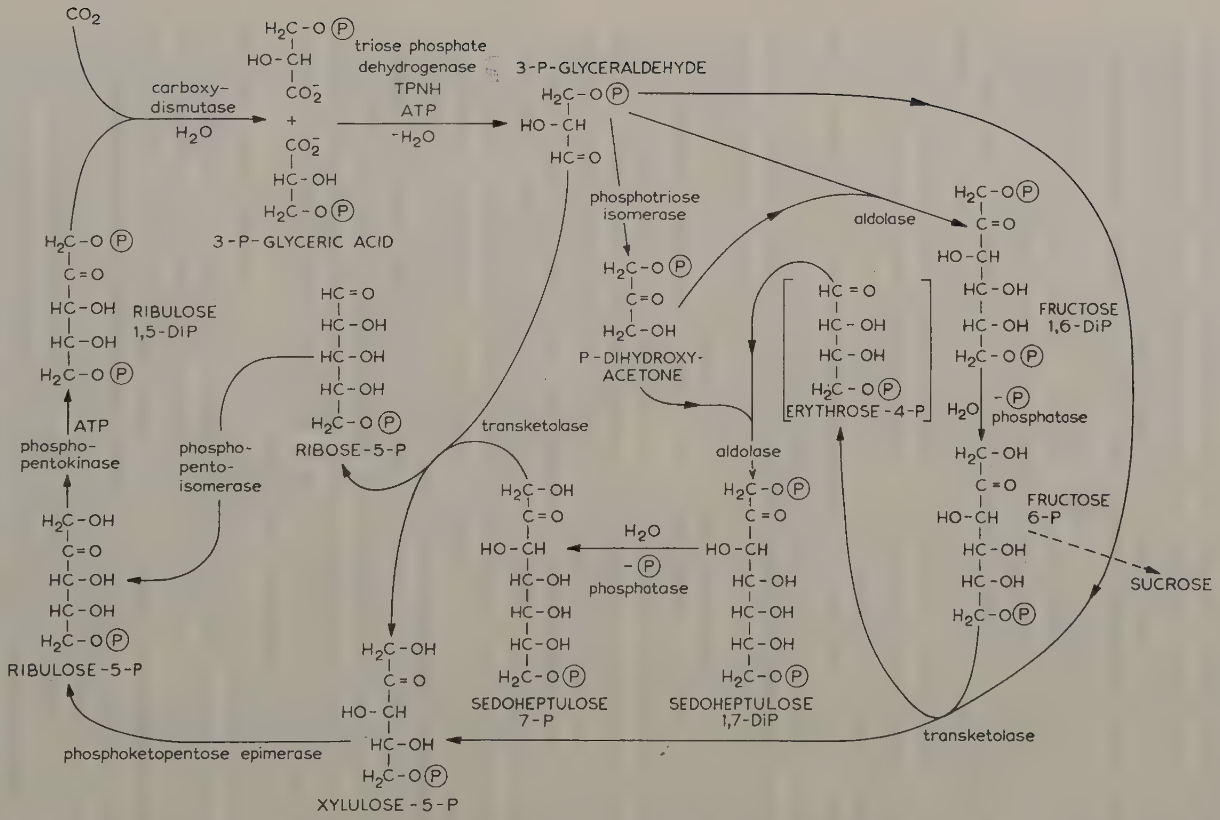

CO$_2$
carboxy-dismutase
H$_2$O
3-P-GLYCERIC ACID
triose phosphate dehydrogenase
TPNH
ATP
-H$_2$O
3-P-GLYCERALDEHYDE
phosphotriose isomerase
P-DIHYDROXY-ACETONE
aldolase
FRUCTOSE 1,6-DiP
H$_2$O
-(P)
phosphatase
FRUCTOSE 6-P
SUCROSE
transketolase
ERYTHROSE-4-P
aldolase
SEDOHEPTULOSE 1,7-DiP
H$_2$O
-(P)
phosphatase
SEDOHEPTULOSE 7-P
transketolase
RIBOSE-5-P
XYLULOSE-5-P
phospho-pento-isomerase
phosphoketopentose epimerase
RIBULOSE-5-P
phospho-pentokinase
ATP
RIBULOSE 1,5-DiP

Fig. 20. The photosynthetic cycle according to Calvin[204, 205].

necessary energy is derived ultimately from sunlight. It is assumed that chemically reactive substances descend from the leaves to the roots, and there absorb (radioactive) carbon dioxide. The labeled reaction products, including malic and citric acids, subsequently travel up (are translocated) to the parts of the plant above the ground, and are transformed there into other compounds. (In this way the notion of Aristotle, that the substance of the plant is derived from the soil, is vindicated!) Fertilization with carbon dioxide, by applying carbonate to the soil, may be of practical value[234]. The uptake of radiocarbon from urea by the roots has also been studied[235].

Plants—especially higher plants—synthesize sucrose easily and rapidly. This important substance, whose biological function is not entirely clear [236, 237], has been called the end product of photosynthesis by Calvin. Labeled sucrose appears in paper radiochromatograms even before fructose or glucose, although after their phosphates[238–240]. Its total quantity always exceeds that of the hexoses. According to Calvin, fructose-1,6-diphosphate gives rise to fructose-1-phosphate, on the one hand, and to glucose-1-phosphate (via two intermediate steps), on the other hand. Finally sucrose is formed from these two compounds by a process apparently involving the intermediate transformation of the glucose-1-phosphate into the uridine diphosphoglucose (UDPG), which was discovered in the laboratory of Leloir[123, 200, 241–248].

Experiments carried out in the dark with canna leaves have shown that it is not free hexoses, but rather their phosphates, which are intermediates in sucrose synthesis. On infiltration with labeled hexoses, labeled hexose phosphates are quickly formed, and, after them, the sucrose. If glucose-1-phosphate were able to react with free fructose and liberate orthophosphate, as is the case with *Pseudomonas saccharophila* (p. 34), the fructose moiety of the sucrose would have to be inactive. This is not the case, however; the two parts of the sucrose have activities of the same order of magnitude. The 'fructose precursor' is formed, therefore, mainly from radioglucose (and vice versa)[249].

The fact that the glucose and fructose which form the sucrose have appreciable specific activities, shows that they do not mix with the pools of the inactive hexoses in the cells; rather, they undergo phosphorylation and further reaction at the cell wall. The labeled glucose as such penetrates into the interior of the cell to a small extent at most.

The free fructose (glucose) of the cells proves to be inactive after infiltration of labeled glucose (fructose). There is evidently no invertase in the region accessible to the newly-formed sucrose. On the other hand, infiltrated labeled sucrose is rapidly inverted; this process is probably extracellular[249–251].

Similar experiments on wheat seedlings have demonstrated that the distribution of radiocarbon within the molecules of the hexose administered is mostly maintained when sucrose is formed; in other words, no appreciable degradation and synthesis of hexose takes place. In this experiment the activities of the two hexose moieties of the sucrose were also nearly equal[252].

The sucrose accumulated in beet roots is formed mostly in the leaves, as had long been suspected by the chemists in the sugar industry, and as has now been proved by experiments with labeled atoms[253–256]. The transport of sugar to the roots takes place against the concentration gradient, and is inhibited by carbon monoxide[257,258]. In the root, part of the sucrose is converted into carboxylic acids, amino acids, and other compounds[255,256].

The velocity of translocation of (labeled) sucrose in soy beans was found to be 1.4 cm/min under certain conditions. Sucrose migrates faster than glucose or fructose. Many other radioactive compounds were used in these experiments, and the effects of illumination (non-existent), temperature, and treatment with 2,4-D were investigated[255,256,259–261]. Similar studies have been carried out with sugar cane[262] and sun flowers[263].

Radiophotosynthetic experiments with tobacco have shown that starch becomes labeled very quickly[208,238] and that the distribution of the radiocarbon in the glucose units of the starch depends upon the conditions[264]. Fructose, glucose and sucrose can all be utilized for starch formation, and also for respiration, *e.g.*, by isolated tobacco leaves[236,237,265,266]. Maltose does not seem to play a role in the formation or degradation of starch [248,267]. The concept of the spatial separation of reaction sites must again —as in the study of sucrose formation—be invoked in the study of starch formation with radiocarbon[237]. The products of the hydrolysis of labeled tobacco starch have been examined[268].

In one experiment it was found that the cellulose isolated from cotton plants three months after injection of glucose-1-^{14}C was labeled only in the 1-position[269]. Other authors, however, found radiocarbon in the 6-position as well[270]. The incorporation of carbon from CO_2 into cotton fibers was followed over a long period of time[271]. After administration of glucose-1-^{14}C to wheat sprouts, 80% of the radiocarbon of the cellulose was recovered in the 1-position, and most of the remainder, in the 6-position[252]. The utilization of radiocarbon from several other monosaccharides for the synthesis of cellulose and of xylan by wheat was also studied. It appears that direct poly-condensation of glucose—perhaps by way of UDPG—to cellulose predominates, and that cleavage of the hexose chain occurs to only a small extent[272–275].

A different situation occurs in the case of *Acetobacter xylinum*, which degrades part of the carbohydrates by way of pentoses. Glucose-6-^{14}C did indeed give cellulose labeled only in the 6-position, and glucose-1-^{14}C, mainly cellulose-1-^{14}C; but when glucose-2-^{14}C was used, approximately half of the activity was found in the 2-position and the other half in the 1,3,4,5-positions of the cellulose[276–279]. The explanation is not yet known. Certain enzyme preparations from *A. xylinum* catalyze the synthesis of cellulose from labeled UDPG[280, 281].

4. Carbon Dioxide Fixation by Bacteria

The analysis of the mechanism of the assimilation of carbon by green plants has provided convincing evidence that the assimilation is not directly coupled to the photochemical primary reaction. Studies of bacterial metabolism and of the citric acid cycle (p. 193) reveal that the fixation of free carbon dioxide and the assimilation of the carbon—in the absence of light—is a very wide-spread property of living matter.

The results of the investigations of bacteria have recently been supported by the use of isotopic carbon. From the standpoint of comparative biochemistry, these results are very important, because they shed light on the phylogenetic development of the capacity to reduce carbon dioxide, with or without light; the most primitive living organisms must have lacked this capacity.

It is apparent that isotopes—especially radiocarbon—offer great possibilities for the study of the life processes of heterotrophic microörganisms. After the introduction of the labeled substance, the body constituents and the metabolic products of the organism are examined for activity[282–285]. Further examples are furnished by the investigations on *T. utilis*, *E. coli*, *S. cerevisiae*, and other microörganisms, which will be discussed in later sections.

Isotopic carbon has proved even more important in the study of CO_2 uptake. The organisms (propionic acid bacteria), in which dark assimilation of CO_2 was first demonstrated[286–288], were subsequently investigated with isotopic carbon[289–294], and the work has since been extended to many other kinds of bacteria. A number of reviews on the utilization of carbon dioxide by microörganisms have been published[295–302].

No sharp boundary can be drawn between heterotrophs and autotrophs. On the one hand, some 'autotrophic' organisms can derive their carbon from CO_2 only in the presence of small quantities of certain vitamin-like substances, *i.e.*, of organic compounds. On the other hand, 'heterotrophic' organisms have been discovered, which do indeed require certain organic nutrients as sources of energy, but which nevertheless obtain the carbon for their body entirely or partially from CO_2. An example of the latter is *Acetobacter suboxydans*, which uses alcohol as a substrate but requires CO_2 for reproduction. When radioactive CO_2 is used, the body substances become labeled, while the acetic acid, which is produced mainly from the alcohol, is practically inactive[303]. Analogous results have been obtained with lactic acid bacteria[304]. Certain purple bacteria, which use organic substances as reducing agents, have been more thoroughly investigated from this standpoint[300, 305, 306]. *Rhodopseudomonas gelatinosa*, for example, effects the reaction:

$$2CH_3CHOHCH_3 + CO_2 \rightarrow (CH_2O)_n + 2CH_3COCH_3 + H_2O$$

Carbon from labeled carbon dioxide is incorporated into the cell material, while the acetone remains inactive[307–309].

In some instances, the energy-producing oxidation of a simple substance proceeds all the way to CO_2. There are cases where the uptake of carbon from CO_2 predominates; this is true of the fermentation of glucose by enzyme preparations from *Propionibacterium shermanii*[284]. In another case (*Clostridium thermoaceticum*), where the overall reaction is $C_6H_{12}O_6 \rightarrow 3CH_3COOH$, CO_2 is consumed as fast as it is produced. The intermediate formation of CO_2 can be detected, and the (equal) rates of formation and utilization of CO_2 can be measured with isotopes[310]. In many complex metabolic processes more CO_2 is produced than consumed; one of these is the fermentation of lactic acid, first to acetic acid, and subsequently to butyric acid, by *Butyribacterium rettgeri*[311]. The following reaction sequence has been proposed, where reactions 2 and 3 compete for hydrogen and do not go to completion (see p. 38):

$$\begin{aligned} CH_3.CHOH.COOH + H_2O &\rightarrow CH_3COOH + CO_2 + 4[H] \\ 2CO_2 + 8[H] &\rightarrow CH_3COOH + 2H_2O \\ 2CH_3COOH + 4[H] &\rightarrow CH_3.CH_2.CH_2.COOH + 2H_2O \end{aligned}$$

In this case again the direct proof that carbon is taken up from carbon dioxide requires the use of an isotope. In general, the oxygen content of the fermentation substrate determines whether a net quantity of CO_2 is consumed or produced, but because of the possibilities of competing pathways detailed predictions are unsafe. The dependence of the rate of bacterial growth on the amount of assimilable CO_2 can be investigated with radiocarbon[312].

In the separate determination of the rates of production and consumption of CO_2, it is necessary to beware of the possibility of exchange reactions, which would make the rates appear too large[313]. In the foregoing experiments with butyric acid bacteria, exchange was excluded, since it was found that the carbon dioxide remained inactive when lactate was fermented in the presence of labeled acetic acid. Another source of error in determining reaction velocities is the possibility that CO_2 formed intracellularly can in some circumstances fail to equilibrate with the medium, and hence may be consumed preferentially[311].

The mechanism of the fixation and assimilation of CO_2 can be derived from the distribution of isotopic carbon in the metabolic products[296]. The mechanism assumed for the fermentation by *Butyribacterium* has already been mentioned[311]. Experiments with radiocarbon have also suggested that the autotrophs *Thiobacillus denitrificans*, *Thiobacillus thioparus* and *Thiobacillus thiooxydans*, like the green plants, incorporate carbon dioxide into the carboxyl group of phosphoglyceric acid by reaction with ribulose diphosphate, and that the PGA is thereafter subject to a reaction cycle similar to the one proposed by Calvin[314–318]. Carbon dioxide fixation by the citric acid cycle may also be important[319, 320] (p. 193). The fast uptake of bicarbonate carbon by *Pseudomonas fluorescens KB* grown on acetate may be interpreted as being due to a reaction with pyruvate to give oxaloacetate or malate[321–323] (*cf.* p. 194 and 196).

For references to the investigations with isotopes on the effect of illumination on bacteria the literature must be consulted[129, 132, 301, 302, 313, 324, 325]. In the case of photosynthetic bacteria, as in that of the higher plants, the primary photochemical reaction can be distinguished from the assimilation of carbon both in time and in respect to the nature of the reaction. In other words, the light does not directly activate either the CO_2 or its reaction partner. From a comparative biochemical point of view, the photochemical primary reaction may substitute for the oxidation of a suitable substrate in the dark[335]. It is an important fact that the two processes of photosynthesis and 'chemosynthesis', *i.e.* synthesis deriving its energy from chemical reactions, may occur side by side in the same bacterium. The enzyme systems involved must then be identical in part. Acetone, for example, seems to be fermented by *Rhodopseudomonas* through similar mechanisms both in the presence and in the absence of light:

$$CH_3COCH_3 + {}^{14}CO_2 \rightarrow CH_3COCH_2{}^{14}COOH \rightarrow CH_3COOH + CH_3{}^{14}COOH$$

The free energy furnished by chemical reactions or by light is evidently used to carboxylate the acetone; the cleavage of acetoacetate does not require any further free energy[324–326] (see p. 214). In any case the photosynthetic bacteria need an oxidizable substrate other than water to produce 'reducing power'.

If the same enzyme systems are operating in both cases, the energy required must be provided in the same form. Bacteria can indeed—like the higher plants—utilize light energy for photophosphorylation. The incorporation of radioactive orthophosphate into ATP has been studied in subcellular particles obtained by sonic treatment of the purple bacterium *Chromatium*[327, 328].

In conclusion, we note the rather uneconomical fermentation processes by which the carbon of CO_2, CO, or organic compounds is converted to methane (methane fermentation)[329–331]. Again the mechanism can be elucidated only by the use of isotopes. The methane fermentation of methanol by *Methanosarcina methanica*, for example, has been represented by Van Niel as

$$4CH_3OH + 3^*CO_2 \rightarrow 4CO_2 + 3^*CH_4 + 2H_2O$$

With short-lived radiocarbon ^{11}C it has been shown, at least qualitatively, that carbon dioxide is actually reduced to methane. Another part of the radiocarbon, however, enters the cell material of the bacterium[332].

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CHAPTER XII

THE PRINCIPAL PROBLEMS OF INTERMEDIARY METABOLISM

B. CARBOHYDRATES

1. Degradation of Carbohydrates

The anaerobic degradation of glucose by living matter, or by its enzymes in vitro, often proceeds according to the well-known glycolytic pathway of Embden, Meyerhof, and Parnas, which leads to pyruvic acid. In alcoholic fermentation the pyruvate is decarboxylated to acetaldehyde, and the aldehyde is reduced to the end-product, ethyl alcohol. In lactic fermentation the pyruvate itself is reduced. The reverse process is believed to differ from the scheme below in three places[2] (*cf.* p. 198).

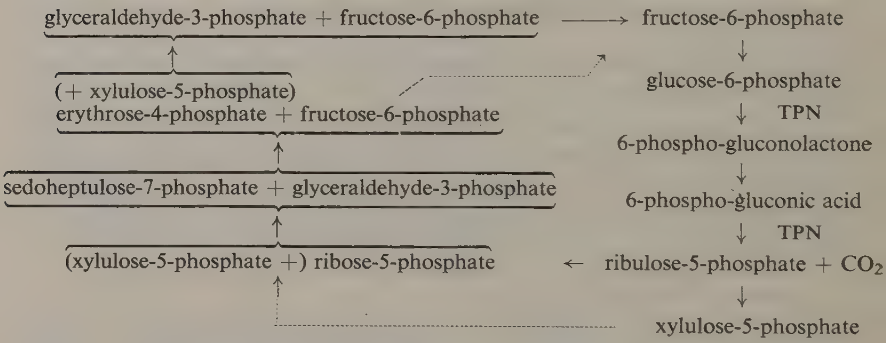
$$\begin{array}{l}
\qquad\text{starch} + \text{PO}_4 \rightarrow \text{glucose-1-PO}_4 \\
\qquad\qquad\qquad\qquad\qquad\qquad\downarrow \\
\text{glucose} + \text{ATP} \rightarrow \text{glucose-6-PO}_4 \rightarrow \text{fructose-6-PO}_4 \xrightarrow{\text{ATP}} \text{fructose-1,6-diPO}_4 \rightarrow \\
\rightarrow (\text{dihydroxyacetone-PO}_4 \rightleftharpoons \text{glyceraldehyde-3-PO}_4) \xrightarrow[\text{PO}_4]{\text{DPN}} \text{1,3-diphosphoglycerate} \xrightarrow{\text{ADP}} \\
\rightarrow \text{3-phosphoglycerate} \rightarrow \text{2-phosphoglycerate} \rightarrow \text{phosphoenolpyruvate} \xrightarrow{\text{ADP}} \\
\qquad\qquad\rightarrow \text{pyruvate} \xrightarrow{-\text{CO}_2} \text{acetaldehyde} \xrightarrow{\text{DPNH}} \text{ethanol}
\end{array}$$

The Embden-Meyerhof-Parnas pathway (glycolysis) (presented according to[1]).

In Section XI, 2 we have already referred to the investigation of some of the partial reactions of glycolysis with labeled atoms. When pyruvic acid is oxidatively decarboxylated, acetyl-coenzyme A is obtained, which can enter the citric acid cycle (p. 193) or the fatty acid spiral (p. 213). The mechanism of the Pasteur effect can also be studied with radioactive carbon[3,4].

In addition to glycolysis, there exist other pathways for the degradation of glucose. These pathways are many and complicated, so that isotope methods offer particular hope of untangling the situation, both with respect to the reactions involved in each of the pathways, and with respect to the extent to which the various pathways participate in carbohydrate degradation in various types of organisms[5–13]. In contrast to glycolysis, pentoses play an important part in other degradative pathways[14]; these pathways serve, therefore, as sources for pentose derivatives in general.

The reaction chain most thoroughly investigated so far is the (aerobic) hexose monophosphate pathway, or HMP (hexose monophosphate shunt). Some of the steps of this pathway are traversed in the opposite direction in the course of the reductive pentose phosphate cycle involved in the photosynthetic assimilation of carbon[15] (p. 172). According to results obtained so far, largely with the aid of radiocarbon, the glucose-6-phosphate is first oxidized to 6-phosphogluconic acid, which is then oxidatively degraded to ribulose-5-phosphate. The reversibility of the latter reaction—which implies a fixation of CO_2 by living tissue through a $C_5 + C_1$ mechanism—has been proved with the aid of radiocarbon[16].


glyceraldehyde-3-phosphate + fructose-6-phosphate
fructose-6-phosphate
(+ xylulose-5-phosphate)
erythrose-4-phosphate + fructose-6-phosphate
glucose-6-phosphate
TPN
6-phospho-gluconolactone
sedoheptulose-7-phosphate + glyceraldehyde-3-phosphate
6-phospho-gluconic acid
TPN
(xylulose-5-phosphate +) ribose-5-phosphate
ribulose-5-phosphate + CO2
xylulose-5-phosphate

A possible scheme for the hexose monophosphate pathway.

The ribulose-5-phosphate is subsequently isomerized by various enzymes, partly to ribose-5-phosphate and partly to xylulose-5-phosphate. In the presence of transketolase there follows a cleavage of phosphorylated xylulose to C_3 and C_2 moieties, the C_2 fragment (active glycolaldehyde) being, however, immediately condensed with another molecule of an aldose to form a ketose. Free (labeled) glycolaldehyde does not undergo the transketolase reaction[17].

When the two-carbon fragment condenses with ribose-5-phosphate, sedoheptulose-7-phosphate is formed, which in the presence of transaldolase yields in turn fructose-6-phosphate and erythrose-4-phosphate. The erythrose-4-phosphate reacts with another molecule of xylulose phosphate to give another molecule of fructose phosphate and a molecule of triose phosphate. Uniformly-labeled triose phosphate and unlabeled sedoheptulose phosphate yield fructose labeled in positions 4,5 and 6; evidently the dihydroxyacetone group of sedoheptulose is transferred to the triosephosphate[18].

Each turn of the cycle causes the loss of one sixth of the carbon by oxidation to carbon dioxide, since three molecules of hexose eventually yield two molecules of hexose, one molecule of triose, and three molecules of CO_2. The whole molecule of hexose would therefore be consumed by six successive cycles. The scheme shown above reproduces the HMP-pathway according to Dickens, with additions based upon later work [18–21].

The methods for distinguishing quantitatively between the HMP-shunt and glycolysis depend upon the fate of carbon atom 1 in the pentose cycle. Thus glucose-1-^{14}C was used as substrate, and the production of isotopic CO_2 was measured. In anaerobic glycolysis only carbon atoms 3 and 4 yield CO_2. To be sure, when the citric acid cycle follows glycolysis, carbon atom 1 can also give CO_2, but only one-sixth of the total CO_2 can arise from this source. The CO_2 produced by degradation via the shunt, on the other hand, arises directly from carbon atom 1. The fraction of the total CO_2 obtained in experiments of this sort, which comes from the carbohydrate reserves of the organism (endogenous substrate), can be determined by control experiments with glucose labeled in various positions [22].

Another method for determining the fraction of carbohydrate metabolized by the HMP-shunt is the following: glucose-1-^{14}C and uniformly labeled glucose are used in parallel experiments, and the specific activities of the C_2 and C_3 compounds are determined. On the assumption that endogenous substrate does not contribute to the formation of these compounds, a three carbon compound (pyruvate or lactate) formed from glucose-1-^{14}C must have the same content of radiocarbon as the substrate if the Embden-Meyerhof pathway alone is operating. If the ratio of specific activities is less than 1, however, this indicates that carbon atom 1 is used preferentially in other ways. The carbon of a C_2 compound formed by glycolysis would have 1.5 times the specific activity of the glucose labeled in the 1-position, since atoms 3 and 4 are lost by decarboxylation when the C_2 compound is formed. Hence, when the C_2 (or C_3) compounds or their derivatives are examined, the deviation of the specific activities from the value 1.5 (or 1) measures the fractional participation of degradative pathways in which carbon atom 1 is lost. Again, the endogenous substrate is disregarded, but parallel experiments with uniformly labeled glucose provide a correction factor. The actual determination of the activity can be carried out, for example, by isolating acetoacetate, which is formed from acetyl-CoA ('active acetate') [23–25] (Chapter XIII).

Several important sources of error are attached, however, to this apparently simple procedure [9, 22, 26–27A]. In the first place, the CO_2 given off can be taken up again and incorporated into carbohydrates, etc. (see below). Secondly, and this is most important, the pentose yields hexose, which can in turn enter glycolysis or the shunt. This hexose, which is degraded in the subsequent reactions, is unlabeled, however, so that the specific activity of the total CO_2 obtained is reduced. Quite generally, the neogenesis of hexose by the pentose cycle—in contrast to the formation of hexose by the reversal of glycolysis—leads to an altered distribution of the radiocarbon[28].

In rat liver the HMP-shunt is of some importance compared to glycolysis; unanimity does not prevail as to its extent[22, 24, 27, 29–33]. The shunt has also been measured in the intact rat[34, 35]. It is important in mammary gland, where glucose-1-^{14}C gives ten times as much labeled CO_2 as glucose-6-^{14}C[36, 37]. In the intact cow also, the pentose cycle or a related sequence plays an important part[38]. Muscle, on the other hand, degrades carbohydrate chiefly by glycolysis[29]. Inorganic phosphate has (in Ehrlich ascites tumor) an effect on the relative contributions of the pathways[39]. Finally, mention may be made of other work on tumor tissue[22, 40–42] and of work on insects[43].

Several reviews[12, 44–47A] are available with respect to the pathways of carbohydrate degradation in microörganisms; these have also been investigated primarily with radiocarbon. (Other work was concerned with carbohydrate degradation by plants[1, 13, 27A, 48–53]). It has been shown that *Penicillium chrysogenum* produces 30 % of $^{14}CO_2$ from glucose-1-^{14}C, but only 0.2 % from glucose-2-^{14}C under the same conditions; the HMP-shunt evidently predominates in this case[54, 55]. More studies have been made of *E. coli*[56–58], *L. mesenteroides*[59, 60], *Pseudomonas*[25, 61–65], lactic[66–69] and propionic acid[70, 71] bacteria, as well as yeast[23, 72–76]; the results vary. The formation of alcohol by yeast extracts from ribose-5-phosphate labeled in the 1-position proceeds by way of hexose-6-phosphate[77].

Finally, conclusions about the mechanisms of synthesis and degradation of carbohydrates can be drawn from the distribution of radioactive carbon in glycogen. This subject will be discussed later.

It has been shown with heavy oxygen that an enzyme from molds, notatin, which oxidizes glucose to the lactone of gluconic acid, catalyzes the transfer of hydrogen from glucose to molecular oxygen[78]. The enzyme is therefore a dehydrogenase, not an oxidase (*cf.* [79, 80]). The reaction catalyzed by the enzyme was

$$C_6H_{12}O_6 + O_2 + H_2{}^{18}O \rightarrow C_6H_{12}O_6{}^{18}O + H_2O_2$$

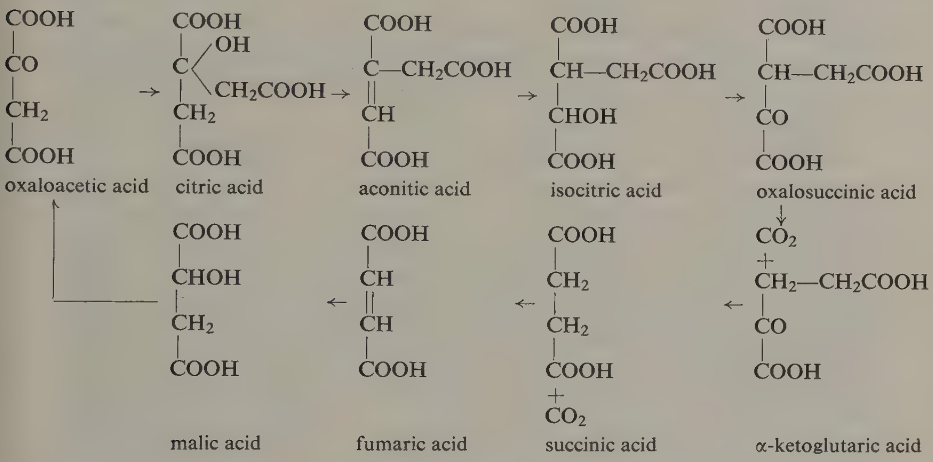
and not

$$C_6H_{12}O_6 + O_2 + H_2{}^{18}O \rightarrow C_6H_{12}O_7 + H_2O^{18}O$$

The absorption and utilization of labeled glucose (and fructose) by surviving rat tissues is inhibited by the metabolic antagonist 2-deoxyglucose[81].

2. The Citric Acid Cycle

Oxidation, which produces twenty times as much energy from glucose as can be obtained by lactic fermentation, proceeds in animals primarily through the tricarboxylic acid cycle (citric acid cycle) discovered by Krebs [82–85].


oxaloacetic acid
citric acid
aconitic acid
isocitric acid
oxalosuccinic acid
CO_2
α-ketoglutaric acid
succinic acid
CO_2
fumaric acid
malic acid

The citric acid cycle.

The occurrence of the citric acid cycle in animal tissues was confirmed in many details by the use of isotopic carbon. After the introduction of labeled acids which are members of the cycle, or of labeled acetic acid, the other members of the cycle are also found to be labeled. The same result is obtained on introduction of substances which can be converted to acetic acid*. *e.g.*, glucose or lactic acid [86]. The location of isotopic carbon in the molecules of the individual components of the cycle, as established by chemical or microbiological degradation, conforms to expectations [46, 87–109]. The isotope method has furthermore permitted the investigation of the relationship to the cycle of a large number of physiologically important substances. Some details will be given in connection with glycogen synthesis (see below).

Whereas labeled substances, which can enter the citric acid cycle, are rapidly altered and finally consumed, this does not hold for other substances, however chemically similar they are. Krebs has said[85] 'It may be argued that the mere occurrence of a set of oxidative reactions in a tissue does not necessarily mean that these reactions have any connection with the main respiratory processes. This argument would carry weight if the tissue were capable of oxidizing a large variety of substances at rapid rates. But this is not the case. The series of reactions composing the cycle is, in fact, the only known set of rapid reactions in muscle by which CO_2 and water, the main end products of respiration, can be formed at significant rates from organic substances.'

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* Strictly speaking, the thio-ester of acetic acid and co-enzyme A (acetyl-CoA) is the metabolite. In the interest of brevity we shall often omit to mention the coenzyme, in regard to acetic as well as to other acids. For the role of coenzyme A *cf.* F. LIPMANN, *Science*, 120 (1954) 855, F. LYNEN and K. DECKER in *Erg. d. Physiol.*, Vol. 49 and the book by K. DECKER, '*Die aktivierte Essigsäure*'; for the fate of CoA in metabolism *cf.* T. WIELAND, W. MAUL and E. F. MOELLER, *Biochem. Z.*, 327 (1956) 85. For the formation of acetyl-CoA, see pp. 165, 189.

In order to label the components of the cycle it is sufficient to incubate the liver homogenate, liver extract, or muscle with radioactive CO_2, or to inject labeled bicarbonate into the living organism. The extent to which CO_2 is taken up into oxaloacetate or malate under specific conditions is a matter of great interest. It appeared that in the presence of ATP the CO_2 was incorporated by pigeon liver extracts chiefly into oxaloacetate, but in the presence of TPNH it was incorporated (with simultaneous reduction) into malate. It was concluded that there exist two parallel mechanisms of CO_2 uptake by pyruvate, or that a common intermediate is formed which yields oxaloacetate or malate, depending on the relative proportions of ATP and TPNH[108,110–113]. The metabolism of malonate—a frequently employed antagonist to succinate—has also been studied with radioactive carbon[114].

A problem of capital importance arose in connection with the formation of succinate from citric acid which was labeled in one of the primary carboxyl groups. It was originally anticipated that 50% of the activity would be lost with the CO_2, since the citric acid molecule is symmetrical, and the enzymatic decarboxylation could proceed just as well by the reaction:

$$HOO\overset{*}{C}CH_2C(OH)(COOH)CH_2COOH \rightarrow \overset{*}{C}O_2 + HOOCCH_2CH_2COOH + \ldots.$$

as by the reaction:

$$HOO\overset{*}{C}CH_2C(OH)(COOH)CH_2COOH \rightarrow HOO\overset{*}{C}CH_2CH_2COOH + CO_2 + \ldots.$$

However, when oxaloacetic acid labeled in the carboxyl group adjacent to methylene was condensed with unlabeled acetic acid in the presence of enzyme, this result very definitely did not occur. The CO_2 actually contained 100% of the radioactive carbon, and the succinic acid formed was completely inactive[90,115,116]. The reverse situation occurred when the citric acid intermediate was synthesized from carboxyl-labeled acetic acid and inactive oxaloacetic acid[94].

Ogston then, in a short communication, which properly caused a sensation, pointed out that citric acid labeled in one of the two chemically

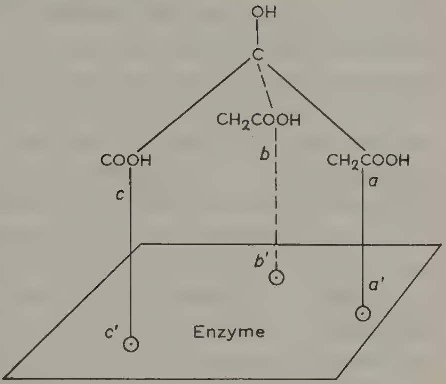

Fig. 21.
Scheme of compound formation between a symmetrical substance and an enzyme (after Ogston).

identical carboxyl groups need by no means appear symmetrical to an enzyme, and therefore, the CO_2 could in actual fact be derived from only one carboxyl group or the other[117]. 'These conclusions (that the symmetrical citric acid must be excluded from consideration as an intermediate, E.B.) seem to arise from the fallacy that, because symmetrical products arising from the *d*- or *l*-form of an optically active precursor cannot be distinguished, therefore the two identical groups of a symmetrical product formed from one optical antipode cannot be distinguished. On the contrary, it is possible that an asymmetric enzyme which attacks a symmetrical compound can distinguish between its identical groups. This power of distinction is illustrated for the case of citric acid in the accompanying formulae* (Fig. 21). *a'*, *b'*, *c'* represent points in the enzyme which specifically combine with the groups *a*, *b*, *c* of the substrate. Evidently, the reaction could occur at *a'* but not at *b'*, or vice versa . . . This argument depends on two conditions: (*1*) that the sites *a'* and *b'* are catalytically different; (*2*) that three-point combination occurs between the symmetrical substrate and the enzyme . . . Since neither is unlikely, it follows that the asymmetrical occurrence of an isotope in a product cannot be taken as conclusive evidence against its arising from a symmetrical precursor.' A more detailed graphical representation is due to Krebs[85].

On this basis, citric acid labeled with a radioelement or with deuterium in only one of the two CH_2COOH groups was prepared chemically from optically active substances, and was introduced into the citric acid cycle. It was confirmed that such citric acid behaved like an asymmetrical molecule. The dehydration of the citric acid to *cis*-aconitic acid always occurs in that part of the molecule which arises from oxaloacetic acid, and the decarboxylation of the ketoglutaric acid to succinic acid involves the carboxyl group which was adjacent to the methylene group of the oxaloacetic acid[118, 119]. The correctness of Ogston's hypothesis was thereby confirmed. Asymmetry of the citric acid type in a more general form was discussed later[120].

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* In the original the analogous case of aminomalonic acid is cited as an example.

In connection with problems of fatty acid degradation (see below), the total amount of the acetate formed in the body of a rat was determined by a method in which a trapping procedure was combined with the isotope dilution principle. The rat was fed labeled acetic acid. At various intervals samples of the acetic acid present in the body of the animal were isolated as N-acetylsulfonamide. From the quantity and activity of this substance, the total amount of acetic acid formed by the animal per unit time was calculated, it being assumed that the active and inactive acids were thoroughly mixed and equally reactive (*cf.* however, p. 215). An acetic acid production of approximately 1 g/day/100 g of body weight was found[121]. A similar method indicated a production of only about one-tenth of this value for higher plants[122].

Extensive experiments on the utilization of labeled acids show that the citric acid cycle takes place in tumor tissue as well[41, 123–126]. After the addition of labeled acetic, oxaloacetic or palmitic acid, for example, citric acid containing the label was isolated from the tumor tissue[127–129].

There is nevertheless, in general, a difference between normal and tumor tissue with respect to the proportion of the energy requirements of the tissue satisfied by oxidation on the one hand, and by aerobic glycolysis, on the other hand. As shown by Warburg, the contribution of oxidation is much less in cancer cells than in most normal—non-embryonic—cells. The reasons for this difference are still in lively dispute[41, 126, 130–133].

With the help of isotopes it has been established that the citric acid cycle plays an important part in insects[43], micro-organisms[12, 85, 134–138A] and plants[1, 13, 27A, 52, 53, 85, 139–142A]. However, at least in the case of micro-organisms, acetate can be oxidized by routes other than the citric acid cycle[143–149]. A variant of this cycle, which has been investigated in *Pseudomonas fluorescens KB I* by means of radiocarbon, permits the synthesis of C_4 compounds (succinic acid) from acetate alone in the presence of catalytic amounts of isocitrate ('glyoxylate cycle'); the 'normal' citric acid cycle operates concurrently in the organism. The glyoxylate cycle permits the synthesis of cell constituents of all kinds from a medium containing carbon in the form of 2-carbon fragments only[27A, 138A, 150–154].

Citric acid fermentation, for example by *Aspergillus niger*, may be based on a sort of fragmentary citric acid cycle. Here too the active acetate seems to condense with oxaloacetate, as was proposed as early as 1919 by Raistrick[46, 155] and confirmed in numerous and extensive experiments with isotopic carbon[46, 156–161]. The oxaloacetate can arise by the Wood-

Werkman reaction, that is, by carboxylation of pyruvate ($C_3 + C_1$ condensation); most of the pyruvate is produced by glycolysis. The accumulation of citrate is due to a block in the Krebs cycle; under conditions where no accumulation of citrate is observed with *A.niger*, the introduction of labeled substrates leads to the formation of citric acid whose carbon has passed through the cycle one or more times, as may be seen from the distribution of the label. In citric acid fermentation by some organisms, including *A.niger*, a $C_2 + C_2$ condensation also seems to occur (see above,[150, 162, 163]).

The citric acid cycle serves not only for the production of energy, but also for the synthesis of intermediates for the production of cell constituents[53, 138A, 164]. This function was further investigated in *E.coli*. After the introduction of radioactive substrates and with the use of the 'isotopic competition method', the origination of two families of amino acids by way of glutamine and asparagine was followed[165] (*cf.* also p. 243).

3. Glycogenesis

Isotope experiments on the liver and muscle of mammals and birds confirm that the synthesis of glycogen from the (labeled) products and intermediates of glycolysis occurs in the main by the reversal of the glycolytic reactions. Synthesis from Krebs-cycle intermediates proceeds by way of pyruvic acid. Other substances are glycogenic if they can be converted enzymatically into intermediates of glycolysis or of the citric acid cycle [166–168].

In the fundamental studies of Wood and others, bicarbonate and simple fatty acids were employed as substrates[106, 169–174]. (The stable isotope ^{13}C was often used in those days.) The labeled material was fed to rats whose glycogen had been depleted by fasting, or injected into them. After several hours the liver glycogen was isolated, hydrolyzed to glucose, and selectively degraded (p. 40). The isotopes in positions 1 and 6, 2 and 5, and 3 and 4 were often obtained together, but checks with chemical degradative procedures have shown that the two carbon atoms of each pair have essentially the same isotope content[175, 176]. The results have been summarized by Wood (Table 8) in a simplified form.

Evidently, two different distributions of the labeled carbon exist. Since labeled glycogen is also formed from bicarbonate, the first question is whether the carboxyl groups of carboxyl-labeled acids (which give the same distribution of isotope as the bicarbonate) are oxidized to carbon dioxide, which is thereafter used for the synthesis of glycogen. Although such reactions must occur, a comparison of the absolute contents of isotope on addition of bicarbonate, on the one hand, and of the acids, on the other hand, (not apparent in the simplified table) shows that they can have played only a very minor role. When the labeled carbon is found in other positions than 3 and 4, such an explanation is entirely out of place, since positions 1, 2, 5, and 6 are only faintly labeled by carbon dioxide (see below).

TABLE 8

THE DISTRIBUTION OF LABELED CARBON IN GLYCOGEN

Substance administered	*Distribution of the isotope*
$\mathrm{NaH\overset{*}{C}O_3}$	$\mathrm{C\ C\ \overset{*}{C}\ \overset{*}{C}\ C\ C}$
$\mathrm{CH_3\overset{*}{C}OOH}$	$\mathrm{C\ C\ \overset{*}{C}\ \overset{*}{C}\ C\ C}$
$\mathrm{\overset{*}{C}H_3COOH}$	$\mathrm{\overset{*}{C}\ \overset{*}{C}\ \overset{+}{C}\ \overset{+}{C}\ \overset{*}{C}\ \overset{*}{C}}$
$\mathrm{CH_3CH_2\overset{*}{C}OOH}$	$\mathrm{C\ C\ \overset{*}{C}\ \overset{*}{C}\ C\ C}$
$\mathrm{CH_3\overset{*}{C}H_2COOH}$	$\mathrm{\overset{*}{C}\ \overset{*}{C}\ \overset{+}{C}\ \overset{+}{C}\ \overset{*}{C}\ \overset{*}{C}}$
$\mathrm{\overset{*}{C}H_3CH_2COOH}$	$\mathrm{\overset{*}{C}\ \overset{*}{C}\ \overset{+}{C}\ \overset{+}{C}\ \overset{*}{C}\ \overset{*}{C}}$
$\mathrm{CH_3CH_2CH_2\overset{*}{C}OOH}$	$\mathrm{C\ C\ \overset{*}{C}\ \overset{*}{C}\ C\ C}$
$\mathrm{CH_3CH_2\overset{*}{C}H_2COOH}$	$\mathrm{\overset{*}{C}\ \overset{*}{C}\ \overset{+}{C}\ \overset{+}{C}\ \overset{*}{C}\ \overset{*}{C}}$
$\mathrm{CH_3\overset{*}{C}H_2CH_2COOH}$	$\mathrm{C\ C\ \overset{*}{C}\ \overset{*}{C}\ C\ C}$

C inactive $\quad \overset{+}{\mathrm{C}}$ weakly active $\quad \overset{*}{\mathrm{C}}$ highly active

In order to explain the position of the label when isotopic bicarbonate is administered, we start with the fact that the oxaloacetic acid formed by the enzymatic fixation of carbon dioxide can be reversibly converted via malic acid to fumaric (and succinic) acids. Since the latter two acids are symmetric, the radiocarbon gets into the other carboxyl group of the oxaloacetic acid by conversion to these acids and then back again to oxaloacetic acid (dicarboxylic acid 'shuttle'):

$$\underset{\text{oxaloacetic acid}}{\mathrm{HOOC|CO|CH_2|\overset{*}{C}OOH}} \rightleftharpoons \underset{\text{malic acid}}{\mathrm{HOOC|CHOH|CH_2|\overset{*}{C}OOH}} \rightleftharpoons$$

$$\underset{\text{fumaric acid}}{\mathrm{HOOC|CH{=}CH|\overset{*}{C}OOH}} \rightleftharpoons \underset{\text{malic acid}}{\mathrm{HOOC|CH_2|CHOH|\overset{*}{C}OOH}} \rightleftharpoons \underset{\text{oxaloacetic acid}}{\mathrm{HOOC|CH_2|CO|\overset{*}{C}OOH}}$$

The same distribution of radiocarbon is to be expected when the carbon of the CO_2 is incorporated directly into malate with simultaneous reduction (p. 194). It is also assumed that the oxaloacetic acid can yield phosphopyruvic acid directly, rather than pyruvic acid, and that the phosphopyruvic acid can form glycogen by a reversal of glycolysis. A high energy barrier is thus circumvented[13, 177].

In any case, decarboxylation of the oxaloacetic acid produces carboxyl-labeled pyruvate or phosphoenolpyruvate. When glucose and glycogen arise from these compounds, carbohydrates labeled in the 3- and 4-positions must be formed. Metabolism of carboxyl-labeled pyruvate by way of intermediate citrate, after oxidative decarboxylation to acetyl-CoA, naturally causes the radiocarbon to be lost altogether (Table 9).

Labeled acetyl-CoA or even acetate can form labeled glycogen only after passing through the whole citric acid cycle—at least in the tissues of higher animals. No net increase in glycogen can occur, since two carbon atoms—but not those of the acetic acid—are lost in traversing the cycle[172, 178–180] (*cf.* however p. 196). Carboxyl-labeled acetate yields carboxyl-labeled citrate, succinate, etc., and finally carboxyl-labeled oxaloacetate, pyruvate, and 3,4-labeled glycogen. The re-entry of the labeled oxaloacetate into the cycle cannot give any different labeling of the glycogen either, since both carboxyl groups of oxaloacetate are lost as CO_2 in the course of the cycle—by decarboxylation of oxalosuccinic and α-ketoglutaric acid.

But when methyl-labeled acetate enters the cycle, the center carbons of succinate, and hence of oxaloacetate, are labeled. Pyruvate formation from this oxaloacetate leads to labeling in the methyl and carbonyl groups and hence to 1,2,5,6-labeled glucose. It is true that re-entry of the oxaloacetate into the cycle gives carboxyl-labeled succinate, oxaloacetate, and pyruvate, and hence additional 3,4-labeling of the glucose. Nevertheless, the isotope content in the 3,4-positions, since it is achieved only after a long series of reactions (two turns of the cycle), during which isotope dilution must occur, should be relatively small. This corresponds to the experimental findings (Table 8). Butyric acid labeled in the 1- (or 2- and 3-) positions behaves like acetic acid labeled in the 1-(or 2- and1-)positions, as is to be expected on the basis of current theories of the metabolism of fatty acids (Chapter XIII).

Although the mentioned possibility of direct synthesis of glycogen does

TABLE 9

REDISTRIBUTION OF RADIOCARBON IN PYRUVATE

Original position of label	*Position of label*		
	After formation of oxaloacetate and passage through the citric acid cycle	*After formation of oxaloacetate and passage through the 'shuttle'*	*After formation of acetyl-CoA and passage through the citric acid cycle*
1 (carboxyl)	0	1	0
2 (carbonyl)	2 + 3	2 + 3	1
3 (methyl)	1	2 + 3	2 + 3

exist, pyruvate (and lactate) are metabolized in part, after formation of either oxaloacetate or acetyl-CoA, via the entire citric acid cycle. This must be true because α- or β-labeled pyruvate (or lactate) yields glycogen with the isotope in the 1- and 6-positions as well as in the 2- and 5-positions. By comparison of the isotope contents of the various positions of the glycogen one can in principle calculate the fractions of the pyruvate (lactate) which have been metabolized along the various pathways[172, 174, 180–182]. In contrast, carboxyl-labeled pyruvate, as has already been emphasized, can never yield glycogen labeled in any positions other than 3 and 4. Table 9, which can easily be derived from the citric acid cycle, makes these relationships clear.

After the occurrence of the HMP-shunt in liver had been demonstrated, the question had to be raised as to how these reactions participate in glycogenesis. It can be determined whether glucose-1-^{14}C yields glycogen-1-^{14}C; according to the pathway of the pentose cycle this cannot occur, but in the actual experiments it has often been observed[183, 184]. The participation of the shunt would be revealed, after the addition of suitable degradation products of glucose, by the appearance of radiocarbon atoms in positions of the glycogen molecule into which they could not enter by a reversal of glycolysis. After incubation of liver slices with labeled CO_2, 5–10% of the radiocarbon was actually found in positions 1, 2, 5 and 6 of the glycogen. (These experiments were apparently more precise than those reported in Table 8). Nonetheless these experiments, as well as those on intact animals, show that the principal pathway of glycogen synthesis in rat liver is the reversal of glycolysis[185–188]. Labeled ribose is a precursor of glycogen in the intact mouse and in rat liver[30, 188, 189].

Some data on the formation of glycogen from odd-carbon fatty acids are contained in Table 8. Later, more extensive work has confirmed these data and led to the conclusion that in the transition from propionate to pyruvate the α- and β-carbon atoms are partially exchanged with one another[172, 180]. A molecule of a higher odd-carbon acid yields, as will be discussed in Chapter XIII, through 'β-oxidation'—repeated, if necessary—one or more molecules of acetyl-CoA and one molecule of propionyl-CoA. The carbon atoms of the acetyl-CoA can appear in glycogen, as has already been described, while the propionyl-CoA is converted first to succinate. The latter enters the citric acid cycle and can thus also take part in glycogenesis (see p. 213).

The specific activities of glycogen fractions of different average molecular weights, obtained from the liver and muscle of rats after feeding labeled substances, are not the same. Evidently endogenous, unlabeled precursors participate in the biosynthesis of glycogen to varying degrees. Nor is there uniform specific activity within the macromolecule; the peripheral glucose residues are renewed most rapidly[190, 191]. A report has been published[192] on the dependence of the glycogen turnover of the brain upon its functional state.

Hydrolysis to glucose has revealed that glycogen derived from galactose-1-^{14}C, like that formed from glucose-1-^{14}C, is labeled in the 1-position. The galactose is evidently converted to glucose (epimerized) without a split in the hexose chain[193]. The mechanism of the reversible transformation, which requires the presence of UTP, has been studied with the aid of radiocarbon, deuterium, tritium, and heavy oxygen[10, 194–196]; intermediary steps probably consist in the formation of glucose-1-phosphate and UDPG[27A, 197–200].

The relationship between galactose and glucose is of very great interest in connection with the formation of lactose[201]. The galactose portion of the lactose is formed easily from labeled glucose[192, 193, 202–207B] by intact mammals, slices of mammary glands, or homogenates. When glucose-1-^{14}C or galactose-1-^{14}C was injected into guinea pigs, both parts of the lactose proved to be radioactive; the radiocarbon was contained primarily in the 1-position[208]. By a suitable choice of the site of injection of labeled acetate, it can either be effected that the glucose and galactose moieties of the lactose have similar activities (when the galactose is synthesized from blood glucose), or that only the galactose is highly active and shows an intramolecular distribution of radiocarbon different from that of the glucose (when the galactose is synthesized from the acetate directly in the udder of a cow)[209–211]. The pentose pathway is important in the utilization of carbohydrates by the cow (p. 192).

4. Respiration in Intact Animals

We shall mention only briefly several experiments in which substances containing radiocarbon were fed to intact animals, and the activities of the organs, of the respiratory CO_2, and of some of the metabolites were determined under various experimental conditions. Substrates investigated included glucose[212, 213], sucrose[214], and galactose and lactose[213]. Work has also been done on ethyl alcohol[215, 216], methyl alcohol[217], carbon dioxide[212, 218, 219], urea[220–222], carboxylic acids[219, 223–225], lipids[226] and amino acids[227–230]. Individual organs, such as muscle, have also been investigated[231]. For experiments on the respiration of the carbon from drug-like substances, *cf.* Chapter XVI.

A procedure for following exhalation continuously has been described (Fig. 22). A current of air is drawn through the chamber holding the animal. The quantity and activity of the expired CO_2 are determined simultaneously by measurements of the infrared absorption and the ionization. The

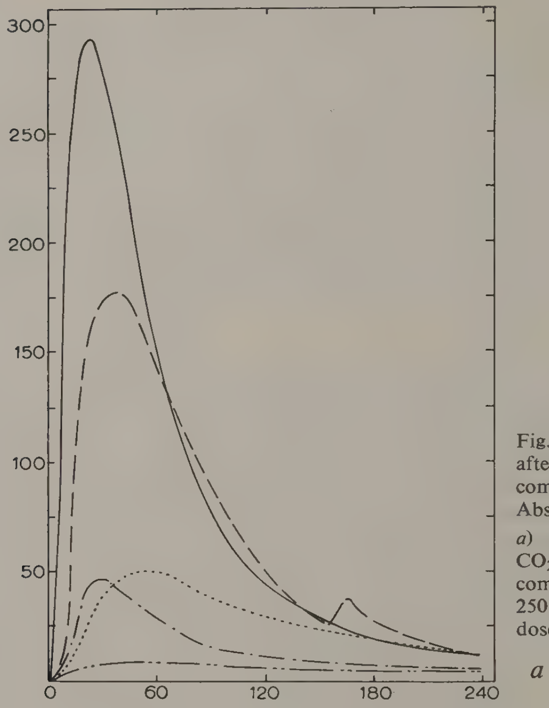
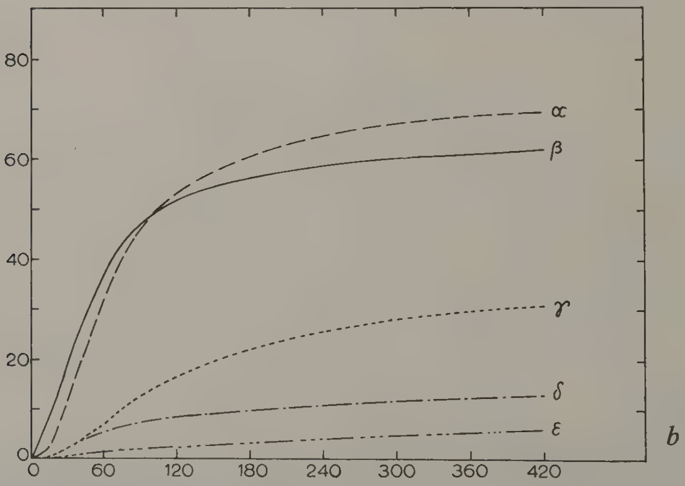


Fig. 22. Expiration of $^{14}CO_2$ after injection of labeled compounds into rats[232]. Abscissa: time in minutes.

a) Specific activity of the CO_2, in mμC/g carbon (recomputed to the basis of 250 g body weight and a dose of 10 μC).



b) Total activity of the CO_2, in percent of the dose.
The labeled compounds are denoted by the same type of curve (solid, dotted, etc.) in both graphs.

α = glucose-U-14, β = acetate-2-^{14}C, γ = DL-leucine-2-^{14}C,
δ = glycine-2-^{14}C, ε = tripalmitin-1-^{14}C (labeled in all palmitate residues).
Note: glucose-U-^{14}C = glucose generally labeled.

ratio of the two values, i.e., the specific activity[232,233], is automatically determined.

The respiration, and hence the energy production, of animals can be determined by injecting $D_2{}^{18}O$ and measuring the deuterium and heavy oxygen contents of the body fluids at various times. Because of respiration, the isotopic oxygen disappears more rapidly than the isotopic hydrogen. The same authors also followed respiration by measuring the speed of incorporation of heavy oxygen from enriched air into body water [234,235]. These methods are based on the assumption that oxygen of respired CO_2 is derived from water rather than from molecular oxygen. This assumption also holds true for plants[236].

In order to draw conclusions about the quantity of exchangeable CO_2 present in an animal from the amount of CO_2 expired per unit time, computations were made on the basis of the concept of an ideal animal consisting of several body compartments, and the results were tested with radioactive CO_2 (see p. 146). It is evident that the analysis of expired air—and of other metabolites—in experiments of this type may be exploited for medical diagnoses[237,238].

5. The Energy Supply of Tissue Cultures

A uniform material, which is not 'diluted' with normal cells, is especially suitable for the investigation of respiration and fermentation in cancer cells. For this reason metabolic experiments with radioelements have been carried out on suspensions of mouse ascites tumor cells. Also, such cells are certainly all in the same state with respect to the supply of nutrients and of oxygen and to the removal of metabolic products. The metabolism can therefore be referred to the number of cells. The 'viability' of ascites tumor cells has been determined by their ability to take up labeled phosphate from the nutrient medium[239].

It is nevertheless advantageous to investigate solid cancer tissue in culture. In the first place, not all types of cancer cells can be propagated in the form of ascites tumors. Secondly,—in the same connection—the cells of cancer tissue cultures may be in a more 'natural' state than the free-swimming ascites cells, which obviously constitute a special case. In the third place, a direct comparison of the metabolism with that of normal tissue is possible in tissue culture. Tissue can be cultivated as a monocellular layer covering the walls of the vessel. Biochemical research on tissue culture has expanded greatly in recent years [239A].

Tissue cultures—and also suspensions of ascites cells—always have very small masses, so that the quantities of metabolic products are also small.

Consequently, for a moderately detailed study of the metabolism advantage must be taken of the sensitivity with which radiocarbon can be detected. The experimental technique consists, then, in incubating the tissue in culture or the suspension of ascites cells for several days in a medium containing a radioactive substrate and thereafter determining the distribution of the radiocarbon among the various chemical fractions obtained from the material. It often suffices to introduce micrograms (fractions of a microcurie) of radioactive substrates[240].

Cultures grown in roller tubes are particularly suitable, since the nutrient medium is kept in constant motion, and bathes the tissue uniformly. The tissues grow in a fairly reproducible manner, are vigorous, and display an active metabolism[241]. 'Hanging drop cultures', in which the tissue spreads on a cover glass and is surrounded by stagnant medium, have also been used for work with radioelements; this method has the advantage that the growing tissue can easily be observed under the microscope.

In a series of investigations with roller tube cultures, labeled glucose was used as the substrate. At the end of the experiment the carbon dioxide (end product of respiration) was washed out of the nutrient medium with a current of inactive CO_2, group separations were carried out with the dissolved substances, and their radioactivities were determined with the gas Geiger counter[242–244].

Among other things, the question whether normal and neoplastic cultures possess enzyme systems for the oxidative degradation of certain radioactive substrates has been investigated by such methods[245–247]. It was found that mesenchyma tissue as well as HeLa carcinoma tissue oxidizes glucose, fructose, mannose, palmitic acid, alanine, tyrosine, and glycine to radioactive CO_2; the oxidation of tyrosine is accelerated by ascorbic acid. Sucrose was not attacked by either mesenchyma or cancer tissue. Fibroblasts (mesenchyma) appear to utilize glycerol better than the cancer tissue investigated.

Glucose is the substrate usually employed to examine the relationship between respiration and fermentation, and in particular the validity of the concepts of Warburg (p. 196) on the relative roles of respiration and of aerobic fermentation in normal and cancer tissues[243, 244]. In both kinds of tissue, the ratio of fermentation to respiration is displaced upward either by an abundance of metabolic fuel (Crabtree effect) or by cultivation under sub-optimal conditions[248, 249, 249A]. Yet it is concluded from the tracer experiments with tissues, as far as they were subjected to suitable tests, that respiration plays a role which is qualitatively different in normal and cancer tissue. The respiration (but not the fermentation) could be suppressed by the addition of cyanide in both normal and cancerous tissues; yet this reagent, within a certain range of concentrations, killed the normal tissue only, and not the tumor tissue[250]. On the other hand, normal tissue (but not the HeLa tissue) can be forced by starvation to give up fermentation in favor of respiration nearly completely[248].

The incorporation of radiocarbon from amino acids and from proteins into tissue in culture has also been studied[239A]. We shall return to some of the results on p. 232.

Moreover, experiments have been carried out with radioactive isotopes on the metabolism of tissue cultures from plants[251–254].

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CHAPTER XIII

THE PRINCIPAL PROBLEMS OF INTERMEDIARY METABOLISM

C. FATS

1. The Degradation of Fatty Acids

Less is known about the degradation and synthesis of fats than of carbohydrates, but largely because of experiments with isotopes considerable progress has been made with regard to the enzyme systems involved[1–12]. Most investigations of fat metabolism with labeled atoms have been carried out on animal systems, but some work has also been done on plants [13–22]. Some important work has been performed with microörganisms; this will be discussed later. Specific methods of degradation for the complete determination of the distribution of isotope in the fatty acids have been worked out[23–26].

The rapid turnover of deuterium-labeled fat in the animal body was observed quite early[27–31]. By administering radiocarbon-labeled acetate to rats maintained in metabolic equilibrium on a fat-free diet, it was demonstrated that saturated liver fat has a half-life of one day, and saturated depot fat, of 16–17 days. The half-lives of the unsaturated fats are 2 and 20 days, respectively[32–36].

It is assumed that fats are always hydrolyzed before being degraded, so that the degradative enzyme systems can attack the free acids. In nature, the high-molecular weight, even-numbered acids (C_{14}, C_{16}, C_{18}) predominate. They are degraded mainly by β-oxidation. Both of the fragments formed—acetic acid, and the fatty acid with reduced chain length—appear in the 'active' form, *i.e.*, as thioesters of coenzyme A (Fig. 23, see p. 193). Soluble preparations have been obtained from liver mitochondria, which catalyse the degradation of the fatty acids[37].

As has been discussed already (p. 189), acetyl-CoA is also produced in the degradation of carbohydrates, by the oxidative decarboxylation of pyruvic acid. The acetyl-CoA produced from fatty acids can either enter the citric acid cycle and be oxidized, or be used for the synthesis of complicated compounds like porphyrins or sterols. The other product of the

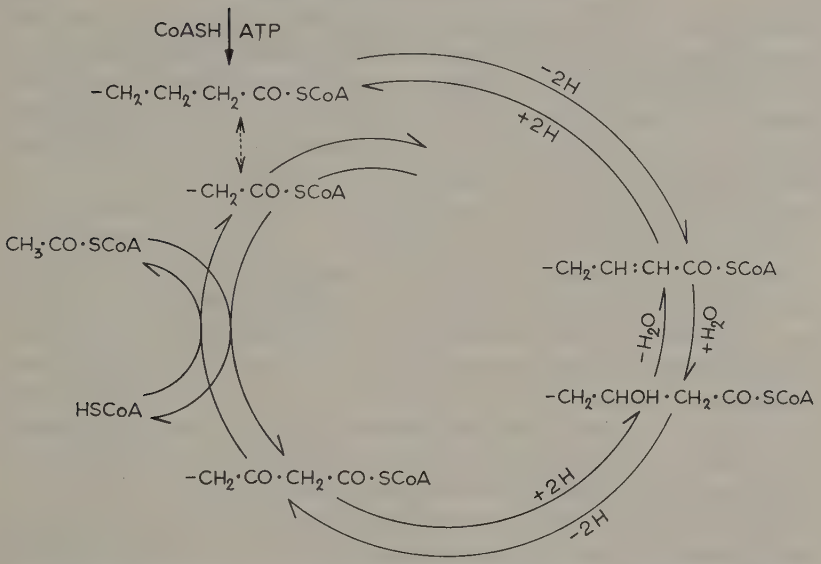
CoASH
ATP
$-CH_2 \cdot CH_2 \cdot CH_2 \cdot CO \cdot SCoA$
-2H
+2H
$-CH_2 \cdot CO \cdot SCoA$
$CH_3 \cdot CO \cdot SCoA$
$-CH_2 \cdot CH:CH \cdot CO \cdot SCoA$
$-H_2O$
$+H_2O$
$-CH_2 \cdot CHOH \cdot CH_2 \cdot CO \cdot SCoA$
HSCoA
$-CH_2 \cdot CO \cdot CH_2 \cdot CO \cdot SCoA$
+2H
-2H

Fig. 23. The β-degradation and -synthesis of fatty acids (simplified), according to Lynen[5].
(„Fatty acid spiral")
CoASH = free coenzyme A
—$CH_3CO \cdot SCoA$ = thioester of coenzyme A

cleavage, the shortened fatty acid, undergoes further β-oxidation; in this way, all the carbon atoms are eventually converted, two at a time, into acetyl-CoA, provided the acid had an even number of carbon atoms to begin with. The complete oxidation of fatty acids is stimulated by the introduction of members of the citric acid cycle, and inhibited by malonic acid[38].

Odd-carbon fatty acids also undergo β-oxidation, but ultimately yield propionyl-CoA. Unlike acetyl-CoA, propionyl-CoA does not condense with oxaloacetic acid, but it can be a precursor of one of the members of the citric acid cycle (succinic acid). The mechanism of the process has been elucidated recently with the aid of radiocarbon[2,3,5,39–41]; it involves a migration of a carboxyl group from methyl malonyl-CoA to succinyl-CoA—a reaction without precedent in chemistry or biochemistry [42]. Propionic acid inhibits the metabolism of labeled acetic acid in rat liver[43,44]. For the distribution of labeled carbon atoms from propionic acid in glycogen, Table 8 and p. 200 should be consulted, and for the synthesis of fat from propionic acid, p. 217. In certain plants, a different pathway of the oxidation of propionate has been demonstrated[45].

The radiocarbon of three specimens of palmitic acid, labeled in the 1-, 6-, and 11-positions, respectively, appeared in the respiratory CO_2 with almost equal speeds after the acid had been injected intravenously into rats. Clearly the stepwise degradation of palmitic acid proceeds very rapidly to completion—or at least to the acetic acid stage—once it has begun. This observation has been explained on the assumption that palmitic acid and the intermediates in its oxidation (*i.e.*, the short-chain fatty acids) remain bound to the enzyme system throughout the entire process[46, 47].

According to the scheme of β-oxidation, 1- or 11-labeled palmitic acid should yield carboxyl-labeled acetic acid, while the 6-labeled compound should yield methyl-labeled acetic acid. Although the carboxyl group of acetic acid is utilized before the methyl group in the citric acid cycle, there was no difference in the rates at which the labeled carbon from the two radioisomers appeared in the respired air; this may be explained by the great rapidity with which the cycle operates[46]. With different experimental conditions, other workers found differences between fatty acids labeled in the odd- and in the even carbon atoms (see[3]).

In the catabolism of fats, the "ketone bodies", acetoacetic acid, acetone, and β-hydroxybutyric acid are also produced; the latter two substances are presumably formed from acetoacetic acid, which is the primary product. Isotopic methods have been most successful in following the production of ketones. Acetoacetate can be formed by condensation of two molecules of (labeled) acetate[48–52]. Since one molecule gives rise to the carboxyl group, and the other to the carbonyl group of the acetoacetate, carboxyl-labeled fatty acids should yield acetoacetate labeled in both the 1- and 3-positions.

$$CH_3\overset{*}{C}O\text{—}CoA + CH_3\overset{*}{C}O\text{—}CoA \rightarrow CH_3\overset{*}{C}OCH_2\overset{*}{C}O\text{—}CoA$$

This has indeed been observed, at least qualitatively. Even carboxyl-labeled butyric acid—which on direct oxidation gives acetoacetic acid labeled only in the carboxyl group—produces acetoacetic acid labeled in both the carboxyl and the carbonyl groups. In this case as well, at least a part of the acetoacetate must be formed by way of C_2 intermediates[48–51]. Butyric acid-3-^{14}C, as expected, also yields 1,3-labeled acetoacetic acid[53]. On the other hand, atoms 2 and 4 of the acetoacetic acid remained inactive when carboxyl-labeled fatty acids were employed[48–51, 54, 55].

Quantitative studies have shown, however, that carboxyl-labeled fatty acids generally produced acetoacetic acid with more radiocarbon in the carboxyl group than in the carbonyl group[1–3, 56]. It is assumed in explanation that acetoacetic acid can be formed not only from active acetate (acetyl-CoA), but also from another kind of C_2 fragment, which is derived only from the terminal ethyl group. (Part of this ethyl group is, of course, normally converted into acetyl-CoA, and enters the pool of active acetate.)

This second kind of C_2 fragment contributes only to the CH_3CO— moiety, but not to the —CH_2COOH moiety of the acetoacetate. On this assumption, those molecules of acetoacetate, whose 'left' halves arise from the ethyl groups of carboxyl-labeled fatty acids, are not labeled in the carbonyl groups[3,5,57–60].

The correctness of this hypothesis was strikingly demonstrated by the use of palmitic acid labeled in carbon atom 15. The ratio of the activities of C-3 to C-1 of the acetoacetic acid then attained the large value of 6, although palmitic acid labeled at C-1, 3, 11 or 13 gave a ratio of 1 or less[61]. Another indication is that the ratio of the activities of the keto and carboxyl groups increases with increasing chain length of the carboxyl-labeled acid; evidently the acetoacetate formed entirely from the acetyl-CoA pool is more predominant when the original chain is long[62]; such acetoacetate, of course, has a 'labeling ratio' of 1.

The conversion of acetyl-CoA to acetoacetyl-CoA is reversible[5,49–51,63,64]. Acetoacetic acid can therefore enter the citric acid cycle by way of acetic acid, and thus be oxidized, as has been demonstrated with labeled carbon[49–51,63,64]. Acetone can be utilized[65–67] by way of (labeled) acetoacetate, provided a source of free energy is available (*cf.* p. 179). The chemical mechanism of the formation of free acetoacetic acid from the coenzyme A thioester has been examined with radiocarbon[68].

Since acetate does not react as such, but rather in the form of acetyl-CoA, exogenous free acetate is not utilized by the tissues (in the Krebs cycle, for example, or to form ketone bodies) as rapidly as biologically-formed acetate, which arises in the active form[49–51]. For this reason, one cannot draw conclusions about the total utilization of acetate from the rate of consumption of added labeled acetic acid. Such considerations cast doubt upon the method mentioned on p. 196 for determining the total formation of acetate.

It is possible, however, to add labeled acetic acid to the tissue, and to compare the utilization in one particular reaction (*e.g.*, oxidation to carbon dioxide) with the simultaneous formation of a standard substance (for instance, acetoacetate). Since both processes start from the same acetyl-CoA, the ratio of the quantities of radiocarbon in the two products is a direct measure of the relative velocities of the reactions. When this procedure was tested with liver slices, it was found that the incorporation of radiocarbon from acetate, lactate and glucose into CO_2 and acetoacetate, as well as into higher fatty acids and into cholesterol, was proportional to the concentration of labeled substrate over a wide range, and independent of the physiological state of the liver. Thus the relative velocities of these reactions, over the range investigated, do not depend on the amount of acetyl-CoA[69–71].

2. Fatty Acid Synthesis

We turn now to the synthesis of fatty acids. The earliest isotopic investigations of fatty acid formation were carried out on microörganisms, including yeast[72–74]. When *Clostridium kluyveri* was grown on carboxyl-labeled acetate, alternate labeling of the fatty acid (1,3,5-positions) was observed; fatty acids were synthesized from acetate in water-soluble extracts[75–79]. In preparative work, randomly labeled fatty acids may be biosynthesized by algae (*Chlorella*) kept in contact with $^{14}CO_2$[80].

The first extensive studies on mammals were initiated by an English group, who took advantage of the special ability of mammary glands to synthesize fat[81]. Carboxyl-labeled acetic acid was injected into a goat, and the fat isolated several days later. The experimental results were interpreted on the assumption that the fatty acids up to and including palmitic acid arose by stepwise addition of two-carbon fragments derived from the acetate, *i.e.*, by a reversal of β-oxidative degradation. The anabolic reaction steps were assumed to be the same as the catabolic steps, except that they proceeded in the opposite direction[82]. The fatty acids produced were labeled in alternate carbon atoms[70]. As expected, part of the tritium of hydrogen-labeled acetic acid was lost during the biosynthesis of fatty acids[83]. In the case of mammary glands, as in that of microorganisms, the synthesis of fatty acids from acetate proceeds even in particle-free extract, provided certain co-factors are present[84–88].

The condensation is comparatively slow, and this fact may be correlated with the relatively high short-chain fatty acid content of milk fat as compared to body fat. On the basis of experiments, in which tritium-labeled fatty acids were fed to goats and cows, it is considered unlikely that the lower fatty acids are formed in a secondary reaction by partial degradation of higher fatty acids[91].

Fatty acid synthesis by condensation of C_2 units has also been observed in rat liver slices and in intact rats and mice[1, 2, 5, 59, 92, 94]. Again fatty acids labeled in alternate positions are obtained from acetic acid with carbon-labeled carboxyl or with hydrogen-labeled methyl[95]. Labeled fatty acids with short chains, incubated in liver slices, are first split into active acetate, and the active acetate is re-condensed to form long chains; for example, if carboxyl-labeled caprylic acid is introduced, fatty acids with alternating labeling are produced[96]. Particle-free systems which synthesize higher fatty acids from acetate can also be obtained from liver[89, 97–105]. Most of the studies demonstrating the central role of coenzyme A in the synthesis of fatty acids have been carried out on liver preparations[5, 6, 105, 106]. Human blood also produces higher fatty acids from labeled acetate[107].

The two reactants concerned in building up the longer chain C_{2n+2}, namely pre-formed fatty acid C_{2n} and acetate C_2, are present as the thioesters of coenzyme A[5, 59, 92, 93]. However, very recent work, some of it carried out with radiocarbon, has led to the conclusion that the condensation of acyl-CoA with acetyl-CoA does not proceed directly. Rather, the acetyl-CoA is first carboxylated to malonyl-CoA, and this combines with the acyl-CoA. Only at a later stage is the CO_2 lost again. Carbon from radioactive bicarbonate is not found in the completed molecule of the fatty acid[108–109A].

Thus the 'fatty acid spiral' (Fig. 23) gives only an abbreviated version of the reductive (synthetic) reactions. Moreover, the two phosphopyridine nucleotides[88–90] and inorganic ions play different roles in degradation and synthesis.

When palmitic acid was formed from carboxyl-labeled acetic acid in the intact mouse, every carbon atom with a label (C-1,3,5, etc.) had the same specific activity[46]. To explain this finding, it was assumed that all the two-carbon fragments used for the synthesis have the same specific activity. This would of course be true in general only if the synthesis of a long chain, once begun, proceeded rapidly to its conclusion in the body of the animal—in contrast to the situation in the mammary gland. In this connection we call to mind the findings in fatty acid degradation (p. 213).

The results obtained on stearic acid with rat liver[110] and intact mice[25] differed from those on palmitic acid, in that the carboxyl group—*i.e.*, the carbon atom incorporated last—had higher activity. It appears, therefore, that preformed (non-radioactive) palmitic acid, which is naturally present in the body in large quantities, serves as a precursor of stearic acid. The same conclusion had been drawn previously from experiments with deuterium on mice and rats[111, 112]. In the intact rat ^{14}C-palmitic acid is built up from ^{14}C-myristic acid and acetate; the carboxyl group does not arise from the myristic acid[113].

After injection of labeled acetate into goats, small amounts of odd-carbon fatty acids are also obtained. When labeled propionic acid is introduced into the isolated udder of a cow, the odd-carbon acids predominate, but labeled even-carbon acids are also found in the butter fat[114]. We have already referred to some studies of the degradation of odd-carbon fatty acids (p. 213).

The thioesters of unsaturated fatty acids are intermediates in the synthesis of saturated fatty acids as was indicated by the example of crotonic acid. The formation of unsaturated depot fat probably proceeds by way of saturated acids. This is in accord with the already mentioned result that the turnover of unsaturated fatty acids is slower than that of saturated fatty acids. It was demonstrated with deuterium as early as 1936 that in the intact mouse stearic acid is converted to oleic acid[115]. The tritium of stearic acid fed to goats is found in oleic acid in the milk[116]. Mammary gland[85, 86] and extracts thereof[88] form unsaturated acids from acetic acid.

The deuterium of D_2O (p. 212) is incorporated into fats *in vivo* (mice) probably by way of carbohydrates and their degradation products[149–152]. On the other hand, deuterium (in heavy water, heavy stearic or oleic acid) is not incorporated by the mouse into linoleic or linolenic acid, substances which the animal cannot synthesize for itself ('vitamins')[117]. Experiments with radiocarbon have shown that linolenic acid is an intermediate in the conversion of linoleic acid to arachidonic acid[118–123].

3. Mutual Conversions of Fats and Carbohydrates

Only brief reference can be made here to experiments on the utilization of the glycerol produced in the hydrolysis of fats[124–126]. The glycerol is phosphorylated to glycerophosphate, and oxidized to dihydroxyacetone phosphate. The carbon skeleton can then be converted along the familiar pathways to glucose or pyruvic acid. Like citric acid (p. 195), glycerol behaves as an asymmetric compound in experiments with isotopes[26, 127–132]. The synthetic enantiomorphic forms of glycerol-1-^{14}C have been applied to the study of this problem[133].

Certain tumor tissues grown in culture appear to utilize (oxidize) radioactive glycerol less readily than do fibroblasts; the ratio of the amount of $^{14}CO_2$ arising from radioglucose to that derived from radioglycerol was much higher for tumor tissue (HeLa) than for the fibroblasts[138].

The conversion of carbohydrate to fat (fatty acid and glycerol) on a fat-free diet takes place on a large scale[1, 139–142]. When radioglucose was fed to mice, 10–15 % of the radiocarbon was incorporated into fatty acids [143]. Glucose is also transformed into fat by mammary glands, and is superior to acetate as a precursor of glycerol[39, 70, 83, 134]. The production of fatty acid from carbohydrate proceeds via pyruvic acid and active acetate; of course, lactate is utilized as well[26, 70, 144–146]. The dependence of lipogenesis upon the extent of glycolysis was investigated with the aid of radiocarbon, and a general parallelism between the two processes was established[1, 5, 147, 148]. The synthesis of fat from fatty acid and glycerol[135] and the distribution of fatty acids among the hydroxyl groups of glycerol[136, 137] have also been examined with radiocarbon.

The conversion of the acid moiety of the fats into carbohydrates proceeds by a more complicated process, and with a lower yield, than the reverse reaction[3]. The entry of acetic acid into the citric acid cycle cannot lead to a net synthesis of glycogen, because each 'turn of the cycle' involves the loss of two carbon atoms as CO_2. Labeled carbon atoms from acetic acid (and hence from higher fatty acids) nevertheless turn up in the glycogen (or alternatively in glycerol[39, 134]), since the carbon atoms lost are not those introduced as acetic acid[153, 154] (p. 199). In some organisms, a net synthesis of carbohydrate from fatty acid can take place through the glyoxylate cycle[155]. Some data on the incorporation into glycogen of carbon atoms from odd-numbered fatty acids are given on p. 198.

We may add a few words on isotopic investigations on the border between biochemistry and physiology. Many tissues other than liver take part in the synthesis and degradation of fat[2, 156, 157]. After labeled acetic acid had been injected into goats, the fatty acids of milk proved more active than those of the plasma; therefore, at least part of the milk fatty acids must have been synthesized *de novo* in the mammary gland[158]. This agrees with the results of the experiments with the isolated organ. However, some part of labeled fatty acids, fed[116] or intravenously injected[159], rapidly enter the milk fat without being degraded first; this applies particularly to long chain acids. When labeled glucose is fed, most of the glycerol carbon in the milk fat of the goat is derived from the material introduced during the last 6 hours[70].

The carbon of radioglucose injected intravenously appeared in the milk of cows to a large extent (56 %) within 48 hours; 46 % was present in the lactose alone (p. 201). Radiocarbon from bicarbonate also entered the components of the milk. Gluconeogenesis was compared to liponeogenesis with a number of radioactive substrates, by determining the ratio of the average specific activities of lactose and of fat after injection of the substrate. Some remarkable results, which admittedly may hold only under the given experimental conditions, are compiled in Table 10[39, 160, 161]. In other experiments under physiological conditions (in the cow), the

TABLE 10

THE RATIO OF GLUCONEOGENESIS TO LIPONEOGENESIS (COW)

Substrate	*Position of label*	*Ratio*
glucose	1–6	15.3
caproic acid	2	9.0
caproic acid	1	1.8
butyric acid	2	3.8
butyric acid	1	2.5
propionic acid	2	8.3
propionic acid	1	2.5
acetic acid	2	0.7
acetic acid	1	0.3
carbon dioxide	–	6.1

formation of fat from carboxyl- and methyl-labeled acetic acid was compared with amino acid formation and with oxidation to carbon dioxide[162].

Experiments employing ^{13}C-acetate and ^{14}C-glucose simultaneously have revealed surprising differences in the capacity of the various species to utilize acetate and glucose for fatty acid synthesis (or for oxidation). It is found, for example, that the ratio between utilized glucose and acetate is higher for the sheep than the rabbit [83,170], and altogether ruminants appear to differ from other mammals[81,171].

We merely mention the investigation of the metabolism of phosphatides by isotopic methods[3,10,32,163–169] (see p. 158).

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CHAPTER XIV

THE PRINCIPAL PROBLEMS OF INTERMEDIARY METABOLISM

D. PROTEINS

1. Introduction

All proteins contain about twenty different amino acids, which are arranged in specific sequences. In view of their complicated structure, the possibility of studying their metabolism with labeled atoms is especially valuable[1]. A great deal of work has been carried out on the reactions of proteins and on the formation, degradation, and mutual interconversions of the amino acids. On the other hand, little is known as yet about the most difficult problem, the mechanism of the synthesis of proteins from amino acids. The present survey deals primarily with protein metabolism in animals, but many important results obtained with microörganisms will have to be mentioned. A review deals with protein metabolism in higher plants[2].

Investigations, which are primarily physiological, will be described only briefly, to the extent that is necessary for an understanding of the biochemical problems. For further details, we refer to the reviews[3-9]. The important possibilities of medical diagnosis on the basis of studies of protein metabolism also cannot be treated here[10,11].

In this chapter, therefore, we propose to make a few remarks on techniques, and subsequently to describe the incorporation of labeled amino acids by organs, cells, and cell components. Moreover, we shall discuss the dynamic state of the proteins and the mechanism of their biosynthesis. Finally, we shall deal briefly with the investigation of the synthesis, the mutual transformations, and the degradation of amino acids by isotopic methods.

According to older concepts, a distinction between endogenous and exogenous metabolism (see also p. 136) of amino acids can be made on the basis that the amino acids once incorporated into (animal) protein normally remain unchanged, and are replaced by amino acids from food only when some of the original amino acid has been consumed (*e.g.*, secreted) or 'worn away'[12]. The validity of this notion was later questioned[13], and investigations with labeled atoms appear to have led to the triumph of the contrary notion of the 'dynamic state' of the body constituents. We may assume, therefore, that proteins—like most other components of the animal body—are in a well-balanced dynamic equilibrium[14, 15]. This leads to two alternatives: either the proteins may be renewed as complete entities, or portions of the molecules—primarily amino acid residues—may be removed and replaced separately. These questions have been taken up again in recent years, and we shall return to them. For the time being, we shall make use of the concept of the dynamic state.

It was early shown that only a part of nitrogen-labeled glycine, which had been fed to experimental animals in physiological equilibrium, was excreted in the feces. Another part replaced unlabeled glycine already present in the body, as will be described below. A third portion of the amino acid underwent chemical reactions (p. 241). The metabolic pool of free tissue glycine is inhomogeneous[16]. In principle, the same results are found with other amino acids. The distribution among the various fractions depends upon the physiological state of the animal and on the dose of amino acid administered[17, 18]. In particular, the incorporation into protein increases if the animal suffers from protein starvation; the release from protein decreases correspondingly[19].

Fig. 24 shows the excretion of isotope after a single dose of labeled glycine as a function of the time. On the other hand, if labeled amino

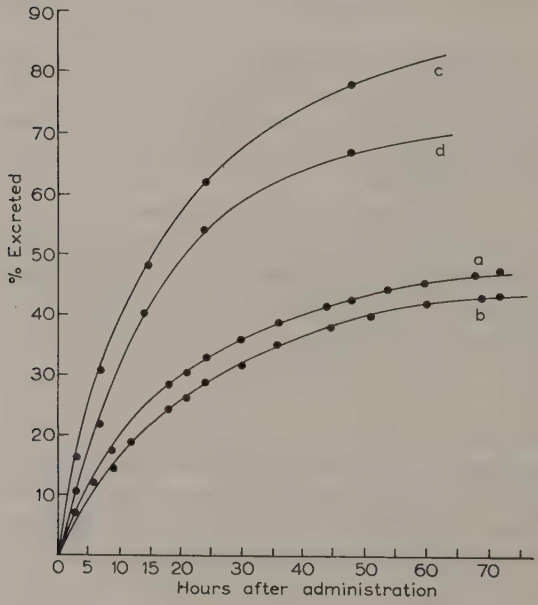

Fig. 24.
The excretion of heavy nitrogen after administration of a single dose of labeled glycine to an experimental subject[20].
Curves a and b: normal diet. Curves c and d: high protein diet.

acid is administered continuously at a uniform rate for a relatively long time, the animal becomes saturated, and the amount of isotope excreted reaches the uptake value. If at this moment—or earlier—the administration of isotope is discontinued, and the isotope content of the excreta followed further, declining curves are obtained.

This rough, empirical procedure yields no information about the chemical changes of the isotope in the tissues. Presumably under certain conditions the amino acid will reach a stationary state which is characterized by equal isotope contents in all the 'dynamic' proteins. The conditions are fulfilled if the amino acid incorporated into all the proteins is taken from the same pool; this implies, for example, that a conversion of other substances into the amino acid cannot occur to different extents in different organs.

The establishment of the stationary state with respect to the isotope occurs at different rates in different organs, and even in different proteins in the same organ. This is proved by analysis at various intervals; in the first period after the administration of the labeled amino acid, a transport between the various organs is also observed[1,21–26]. In conjunction with these investigations, the speed of protein synthesis in the whole organism and the sizes of the amino acid pools have been estimated[20,27–31]. In the isolation of the proteins, contamination by small quantities of relatively active materials must be avoided[31,32]. Amino acids and peptides are sometimes linked to proteins by other than peptide bonds, or even by adsorption[33–42,48]. When carboxyl-labeled amino acids are used, one criterion of real incorporation is assumed to be that ninhydrin releases radioactive CO_2 from the protein only after hydrolysis with enzymes or with acid[37,38,43]. Unfortunately, this criterion is not always reliable[44]. The best proof of actual incorporation of the amino acid is the demonstration of the presence of radioactive α-peptides after partial hydrolysis [45–47] (p. 233).

2. The Incorporation of Amino Acids by Cells and Tissues

All the naturally-occurring amino acids are incorporated into tissue protein; none of them has been observed to exhibit specific peculiarities in this respect. Examples are the incorporation of carbon-labeled glycine[48], tyrosine[48] and other amino acids[23] by the rat, and of ^{35}S-methionine by various species[49–52]. Valine, glycine, lysine, tyrosine, methionine, glutamic acid and glutamine are rapidly incorporated into casein and lactalbumin by goat mammary gland[53,54]. From the practical standpoint, it is well to employ amino acids which do not readily undergo side reactions (*e.g.*, valine or leucine).

Occasionally the incorporation of 'unnatural' amino acids has been attempted. Negative results were obtained with α-aminoadipic acid[48] and alloisoleucine[55], but positive results with ethionine[5, 56–58] and fluorophenylalanine[59, 60]. Selenomethionine can replace the essential amino acid methionine in *E. coli*, and can be recovered from the cell protein after hydrolysis; the hydrolysate contains no trace of methionine, as was demonstrated in a very sensitive manner by growing the organism on a medium containing $^{35}SO_4$[61, 62]. Cattle incorporate labeled norleucine into casein[63]. Experiments on the inhibition of the incorporation of labeled amino acids by unlabeled amino acid analogues (antagonists) have been reported, and the mechanism of the phenomenon has been discussed[5, 60–62, 64–69].

If a cell is to synthesize protein, it must contain the required amino acids in sufficient concentrations. In many cases the amino acids are first transported into the cell (p. 129) by selective processes which require free energy, and which are inhibited by cyanide, dinitrophenol, iodoacetate, or other metabolic poisons[5, 9, 70–73]. The individual acids are concentrated to different extents in a specific manner. For example, the active transport of glycine is pronounced. A system useful for studies consists in a suspension of cells of Ehrlich ascites carcinoma, which take up radioglycine with a half-time of only 3 minutes at a temperature as low as 28°; the influx and efflux (p. 129) can of course be measured separately[74–77]. A similar system is erythrocytes[78]. The concentration of an amino acid in the cells changes when the concentration in the medium changes; the amino acids can often be washed out to a large extent with saline. On the other hand, in some microörganisms, like yeast[79] and *Staphylococcus*[80], part of the amino acid concentrated in the cells appears to be irreversibly bound[79].

All the essential amino acids must be present in sufficient quantities if protein is to be synthesized *in vivo* or *in vitro*. For example, the growth of ascites cells can be limited by restricting the amount of glutamine[81]. All the essential amino acids must be available simultaneously[82]; they are of course incorporated at rates proportional to the contents of the individual amino acids in the particular protein.

The synthesis of protein is demonstrated most clearly by an increase in the quantity of protein. Yet protein molecules can be formed *de novo* in the absence of net synthesis (dynamic state). *A priori*, it is not certain whether the renewal of the amino acids in the protein must (like the production of new protein) occur at rates proportional to the fractions of the amino acids in the protein[21, 83]; an 'exchange' of individual amino acids in proteins, mainly of bacteria, has been reported.

The work of Gale is especially important[66, 84–90]. Certain strains of *Staphylococcus aureus* require a fairly large number of essential amino acids. Some recent experiments on the incorporation of labeled amino acids were carried out with *Staphylococci* whose cell structure had been broken down by sonic vibration. After the destruction of the cell walls, the fragments no longer contain pools of amino acids. Thus the experimental conditions are well defined. The fragments are still able to carry out a wide variety of enzymatic reactions; for instance, they can still incorporate amino acids.

When the synthesis of protein is interrupted by withdrawing essential amino acids, or by preventing the incorporation of one of the amino acids by use of an antagonist, uptake of other labeled amino acids by the fragments still continues. Labeled glutamic acid, for example, is incorporated even when protein synthesis is stopped by removing aspartic acid, or by adding chlorophenylalanine (an antagonist for phenylalanine). Energy is required for the uptake of a single amino acid, just as it is for protein synthesis (see below). The energy may be made available in the form of ATP and hexose diphosphate. Ehrlich ascites carcinoma incorporates radioactive lysine, leucine and valine *in vitro* even when the uptake of phenylalanine is completely blocked by antagonists[91].

For the incorporation of amino acids the presence of certain nucleotides is required. According to Gale, the cell fragments no longer synthesize protein or 'exchange' amino acids if the nucleic acids are extracted, or destroyed enzymatically. The activity of the fragments can be restored by adding RNA or DNA from *Staphylococci*, or, more strikingly, certain nucleotide preparations of low molecular weight. One difference between the processes considered by Gale as total synthesis of protein and 'exchange' of individual amino acids lies in the fact that the two processes are inhibited by different antibiotics; for example, synthesis is blocked by chloramphenicol, and amino acid exchange by penicillin. Gale has called the conditions under which synthesis occurs the 'condition 2', while 'exchange' of individual amino acids is observed under 'condition 1'.

However, the discussion of this type of 'exchange' had to be reopened when it was found with (non-disrupted) *Staphylococci* that labeled amino acid is incorporated to a large extent under condition 1 into cell wall substance, and that this substance is not separated from the protein in the procedure originally used (precipitation with hot trichloroacetic acid). Cell wall material is also contained among the fragments of disintegrated *Staphylococci*. Therefore, the question whether incorporation by exchange exists cannot yet be considered as settled[92–95].

Many values for half-lives of different kinds of proteins have been reported in the literature. Not all these values are meaningful[5, 96]. An important source of errors lies in the fact that insufficient attention may have been paid to the participation of endogenous amino acids in protein synthesis[97] (see below). To some extent, errors of this type can be avoided if the results of parallel experiments with a sufficient number of different amino acids are compared.

Work has been done with serum proteins. When a single dose of methionine was injected intravenously into rabbits, the maximum specific activity of the serum proteins was attained after about 5–7 hours[50, 51, 98]. The briefness of the interval is explained by the fact that the labeled amino acid disappears from the animal quite rapidly. A much longer period would be required to reach a (much higher) maximum activity, if a constant, uniformly-labeled pool of amino acid were produced by continuous (or frequently repeated) injection. In the latter case, the labeling of the protein would take place with the same velocity as that with which the label would be lost after administration[23, 97, 99–102]. Different results are obtained if the amino acid, or protein, is given orally instead of intravenously.

The biological half-life of a protein can be determined by means of the loss of radioelement from the molecule, if the protein was labeled within the animal by administration of the labeled amino acid, and the radioactivity of the protein is measured at time intervals after the radioactive free amino acid has disappeared. Fig. 25 shows the decrease in isotope content of serum proteins which had been labeled in this way with ^{15}N-glycine[30]. The fibrinogen of dogs has been labeled by a single feeding of ^{35}S-yeast[99]. This procedure entails the disadvantage that other proteins become labeled

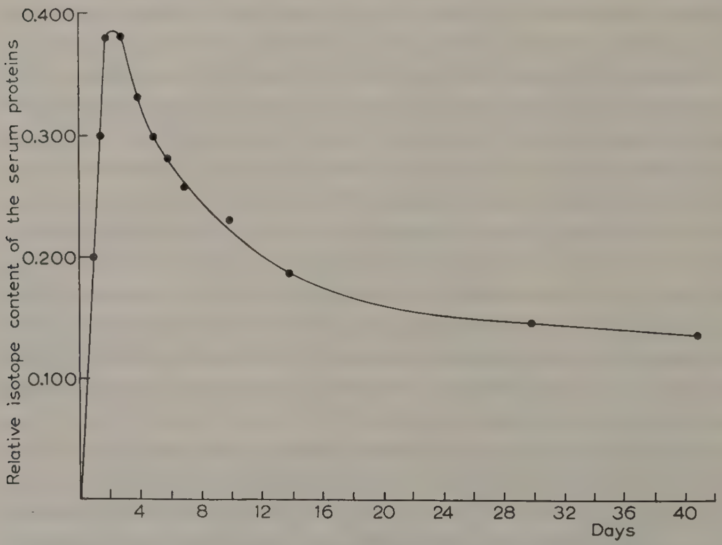

Fig. 25. Time course of the isotope content of the serum proteins of a human subject given ^{15}N-glycine for two days.

as well; labeled amino acids are later released from these other proteins, and then become available for incorporation into the protein under study (*cf.* Section XVI, *1*). Since the various amino acids must pass through pools of different sizes before re-incorporation, different half-times of the specific activity are observed with different amino acids[1,100].

More reliable results are obtained, therefore, by producing the labeled protein in a donor animal, and injecting this protein into a second animal [25,103–106]. In this manner, it is possible to separate and purify the serum proteins before injection[26,29,50,51,106–111]. In Section XVI, *1*, where the subject will be treated from an immunological standpoint, we shall discuss the fact that labeled serum globulin disappears from the circulation by a first-order process. The same is true of serum albumin[100,118]. The degradation of tritium-labeled serum proteins of high specific activity can be followed for an especially long time[112]. According to Maurer, it is possible to use the quantity of labeled amino acid (^{35}S-methionine) released in the degradation of labeled serum protein as a measure of the extent of serum protein degradation at a given moment[101].

It is only immediately after the infusion of labeled protein that a more-rapid-than-exponential decrease in the specific activity of the protein is observed; probably intravascular and extravascular protein are mixing during this period[100,101]. The production of fresh serum protein occurs in the tissues, in the case of albumin particularly in the liver; the molecules are renewed *in toto*, and no amino acids are exchanged[15,100]. One blood protein, i.e., the globin of hemoglobin is fundamentally different from serum proteins in that it is not in a dynamic state (p. 234) but is degraded only after the death of the erythrocyte; a constant life time (although with considerable variations) must therefore be assigned to this protein.

Independently of the ^{15}N-amino acid used in the determination, half-lives of several days have been reported for the plasma and liver proteins of the rat; muscle protein is quite inhomogeneous with respect to nitrogen turnover[1,5,17,21,22,27,113–116]. All half-life values vary with experimental conditions[5,15,117]. The half-life of the serum proteins of mammals increases with the size of the animal[50–52]. The effect of physiological conditions on the proteins (and nucleoproteins) of brain[119–126] and on the proteins of muscle[127,128,130] has been extensively investigated.

As we have stated, the incorporation or release curve is not always exponential; Tarver has offered the following possible general explanations[5]: (*1*) The protein is inhomogeneous. (*2*) The concentration of isotope in the amino acid pool is not negligible, so that re-utilization can occur. (*3*) Barriers exist between the protein in various spaces of the body. (*4*) The mechanism of labeling is complicated. The first explanation surely holds in the case of all insoluble tissue proteins, and the second, for all proteins which are labeled by feeding radioactive amino acids to the experimental animal itself. The third possibility, as has been explained, holds for serum proteins immediately after transfusion, and the fourth applies, for instance, in the interconversion of sulfur-labeled amino acids.

The incorporation of labeled amino acids into tissue slices—liver, spleen and bone marrow—has also been observed[6,7,43,131–134]. Over a certain range, the quantity incorporated seems to be proportional to the logarithm of the concentration of the amino acid[43,131]. However, in an attempt to establish quantitative relationships, the active transport of the amino acids into the cells must be taken into account; the extent of this transport depends upon the amino acid, but is usually doubtful, so that the concentration at the point of incorporation is not known (p. 228). Liver slices utilize the radiocarbon of serum albumin for the synthesis of globulin, for gluconeogenesis, and for oxidation; the albumin is presumably first hydrolyzed[135] (p. 238).

Cell suspensions, *e.g.*, of ascites tumor[136–138] (p. 235), duck erythrocytes (p. 234), and reticulocytes[139] (p. 268) have also been employed in studies of incorporation. In experiments with cultures of various animal tissues, it can be established whether the tissues are capable of the autonomous synthesis of well-defined, soluble proteins[140–142*a*]; the experiments also provide information about the chemical reactions of amino acids and demonstrate which amino acids are essential for each type of tissue[143,144]. The incorporation of radioactive lysine by regenerating single nerve cells has been measured[129].

The rates of incorporation of labeled amino acids into rapidly-growing (embryonic, juvenile, regenerating, or tumor) tissue have been determined *in vivo* and *in vitro*, and compared with the corresponding rates in normal tissues[34,35,38,145–158]. A table showing the incorporation of amino acids into proteins, both *in vivo* and *in vitro*, has been compiled by Borsook[6] a number of years ago.

3. The Incorporation of Amino Acids by Cell Components

We have reported some experiments with broken *Staphylococcus* cells on p. 229. Cell-free homogenates (of liver) also incorporate amino acids into proteins, and synthesize new protein. However, the rate of incorporation is initially less than that with intact cells, and moreover it falls off more rapidly. Experiments have been performed with glycine[6,35,36], lysine[6,43], leucine[6] and methionine[5,49]. Further data have been obtained with the protoplasts obtained by the action of lysozyme on bacteria[160–164].

The isolation of radioactive peptides from the protein hydrolysates has demonstrated unequivocally that the amino acids are actually incorporated into the proteins[167]. Many workers in this field have found that incorporation is inhibited by respiratory poisons and by anaerobiosis[5,32] (*cf.* also pp. 228 and 240).

When labeled amino acids are administered to rats, and the liver cells are fractionated, the highest specific activity is found in the microsomes[165–179]. The microsomes can be further fractionated by treatment with salt solutions of various ionic strengths and pH-values, or by extraction with deoxycholate. The protein fractions associated with the largest amount of ribonucleic acid exhibit the most rapid amino acid metabolism, and hence, the greatest incorporation of labeled amino acids[32,168,169,180,181] (*cf.* p. 230). These findings support the hypothesis which assigns RNA a leading role in protein synthesis (see below and Chapter XV). The high activity of the microsomes has also been demonstrated in plants, *e.g.*, with regard to the incorporation of amino acids into the protein of tobacco leaves[182].

After suitable incubation of cell homogenates with labeled amino acids *in vitro*, the microsomes again prove to be the most active fraction[167,183]. When the individual fractions are incubated separately, the microsomes incorporate labeled amino acids only in the presence of a source of energy and of the soluble cytoplasmic enzymes. Mitochondria can serve as a source of energy (in the presence of glucose and co-factors) since they engage in oxidative phosphorylation[184]. Incorporation has, however, been observed even in a system consisting of microsomes, a soluble protein fraction and co-factors, including ATP and GTP[32,185–192]. The microsomes do not need to be intact; after the microsomal membrane in homogenates of Ehrlich ascites cells had been removed by treatment with deoxycholate, the remaining nucleoprotein particles incorporated amino acids particularly rapidly, provided co-factors and soluble proteins from the cell-sap were present[154,193]. Similar results have been obtained with particulate fractions from pea seedlings[194,195]. The incorporation of labeled amino acids into the protein of chloroplasts has also been reported[196]. On the other hand, despite some reports to the contrary, a true *de novo* synthesis of protein by soluble systems has not yet been demonstrated. But in the presence of threonine and arginine an extract of pigeon pancreas can form amylase from a pro-enzyme without incorporating labeled glycine or tyrosine[197,198].

Isolated cell nuclei from hog kidney[199] and calf thymus[200] incorporate labeled amino acids into proteins[201–202a]; the nuclei themselves provide the necessary energy[200,203,204]. If the proteins are subsequently separated, most radiocarbon is again found in the fraction richest in RNA[200]. Experiments on the giant alga *Acetabularia mediterranea* (p. 265) have shown, however, that the nucleus is not essential for protein synthesis in the cytoplasm; when the algae are cut in two, the incorporation of amino acids into proteins continues for weeks, both in the portion which contains the nucleus, and in the other portion[202, 205]. Autoradiography is a useful tool for the examination of cell nuclei[206–208] (p. 266).

4. The Dynamic State of Proteins

We have cited many examples of proteins which are in a state of dynamic turnover. On the other hand, the abundant protein, collagen, renews its amino acids extremely slowly; thus collagen does not greatly contribute to the dynamic state of the body proteins. In the case of the rat, the average life-time of collagen exceeds that of the animal[209–213]. Ascorbic acid has some effect on the rate of incorporation of labeled glycine into collagen [214–216]. The possibility must be considered that other structural proteins also participate in the dynamic state to only a slight extent. It has been established that some of the structural proteins exchange only slowly (p. 231). Of course, these proteins are harder to define than the serum proteins or the enzymes.

Those proteins, which are irreversibly secreted, undergo dynamic exchange either not at all or only to a small extent. This is true of the globin deposited in the erythrocytes[217–220] (p. 271); the amino acids are, to be sure, re-utilized after the death of the cell. It also holds for the proteins 'exported' in the eggs[214, 221, 222] and in milk[54]. It is probably true of keratin, which has been studied in mice, rats, sheep and rabbits with ^{35}S-amino acids[26, 223–227]. The antibodies in passively-immunized animals (Section XVI, *1*) and the Bence-Jones protein (p. 238) are not renewed.

Viruses constitute a special case. In intact tobacco leaves, tobacco mosaic virus is in a dynamic state even after the maximal (saturation) concentration of virus has been reached[228–230]. On the other hand, tobacco mosaic virus incubated with leaf homogenates does not become radioactive in the presence of radioglucose[231] or labeled amino acids[182].

Monod's studies on micro-organisms have stimulated renewed interest in the dynamic state. When *E. coli* was grown in the presence of ^{35}S -amino acids, and the adaptive enzyme β-galactosidase was afterwards induced in an unlabeled medium, the enzyme proved not to be labeled. On the other hand, when labeled *E. coli* containing radioactive galactosidase was kept in an unlabeled medium, no loss of isotope from the enzyme or from the cell protein was observed. The conclusion drawn was that neither the enzyme nor the cell protein is in a dynamic state; on the contrary, the protein was assumed to be formed irreversibly, and to remain in a static condition[232, 233]. Similar results (also on *E. coli*) were obtained by Spiegelman with radiocarbon[234, 235].

The conclusions of Monod and Spiegelman were confirmed by other methods. In one study, *E. coli* was grown in the presence of labeled glycine, a precursor of purines and proteins, and the *de novo* synthesis of labeled purine suppressed by the addition of unlabeled purine. The cells were transferred to a similar medium, which was unlabeled, and contained no purine. After a further period of growth, the purine was found to contain only a little radiocarbon. Since in the degradation of protein a purine precursor, namely glycine, would be produced, it was concluded that such a degradation took place only very slowly[236].

In view of the similarity of the fundamental processes in all living beings, such experiments cast doubt on the genuine existence of a dynamic state for cell proteins in other organisms including the vertebrates. The presumed dynamic state might be only simulated by the loss of protein through attrition and secretion, on the one hand, and by compensating processes, on the other hand. The renewal of protein would be, so to speak, a physiological rather than a biochemical process. The observed difference in protein metabolism between microbes and multicellular animals would then be due to the facts that the microbes do not replace perished cells in the same sense as higher animals, and that the secretion of protein by the microbes is limited[233].

It cannot be excluded, however, that a dynamic renewal of cell proteins is concealed by a preponderance of fresh synthesis in an organism growing as rapidly as *E. coli*[235]. Some liberation of labeled arginine[237] and of sulfur-containing amino acids[238] has indeed been demonstrated in growing *E. coli*[239]. When *Pseudomonas saccharophila* is labeled with radiocarbon, the amylase induced later does contain radiocarbon[240]. In resting yeast, the dynamic state is much more pronounced than in growing yeast[235, 241]. In non-growing *E. coli* the degradation and resynthesis proceeds at a rate 4–5 % per h; this turnover cannot be attributed to lysis[242].

Multicellular organisms cannot usually be transferred suddenly from a labeled medium to an unlabeled medium. However, this can be done with Ehrlich ascites tumor cells. Moreover, lysis and secretion play only a small role in the separated cells which multiply in suspension. Labeled cells were incubated with a buffer *in vitro* or—enclosed in a cellophane bag—*in vivo*. In both cases the cells released labeled amino acids, and the specific activity of the protein decreased.

In the *in vitro* experiments the inactive amino acids, which replaced the active amino acids, came from the intracellular pool. The conclusion was that the cells were indeed in a dynamic state[244–246]. Experiments on liver and kidney tissue from rats were interpreted similarly; however, the relationships are quite complicated in these cases, and the results, like those with ascites tumor, can be evaluated qualitatively more easily than quantitatively[247]. The continuous exposure technique (p. 230) has also led to the conclusion that liver proteins are in a dynamic state[102,248]. A dynamic state in a non-multiplying animal cell was demonstrated unambiguously in experiments *in vitro* with macrophages from the peritoneal cavity of the rabbit[243].

On the whole it appears that a degradation and re-synthesis of proteins in higher animals does occur without destruction of the cells. But this dynamic process is extremely slow in the case of some proteins, and it is often uncertain to what extent the individual proteins participate. We may conclude that Folin's distinction between exogenous and endogenous metabolism must indeed be modified and re-interpreted in view of the work with labeled atoms, but that it retains a certain amount of validity in a quantitative sense[212,223,249]. The fundamentals of the dynamic state of protein have been discussed from various points of view in review articles [30,203,250].

5. The Mechanism of Protein Synthesis

The site of synthesis of 'transportable' proteins has been investigated with the aid of isotopic tracers. The liver is especially active in rapid protein synthesis (p. 231). Labeled fibrinogen, serum albumin and serum globulin are formed when the intact, isolated rat liver is perfused with blood containing labeled amino acids, or even $NaH^{14}CO_3$[1,49,132,251,252]. Serum globulin is produced even after hepatectomy, however[49,253–255]. Labeled amino acids are incorporated into the various γ-globulin fractions at different rates; it is assumed that these different fractions are produced by different kinds of cells which are not uniformly distributed in the tissues of the organs[133]. Experiments have demonstrated that ^{32}P-vitellin is produced in the body of the hen—probably in the liver—and carried by the blood to the eggs in the ovary[256]. The amino acids used to form milk proteins are derived from the plasma; on the other hand, the amino acids in the plasma proteins are hardly used at all for the synthesis of typical milk proteins like casein, lactalbumin or lactoglobulin [53,54,257–260a].

The mechanism of protein synthesis from amino acids has been the subject of a large number of studies with labeled atoms[7,32,85,134,235,261–265]. The two main hypotheses are the template theory, and the theory of stepwise synthesis through intermediates. According to the template theory, which is put forward, *e.g.*, by Borsook, Brachet, Haurowitz, Lipmann and Spiegelman, the amino acids (or simple derivatives thereof) are taken up in a definite order on a sort of negative mold (template), where they form compounds between themselves, and are released as the finished protein. The theory of stepwise synthesis, which is preferred by Steinberg, Vaughan, and Anfinsen, assumes that the amino acids are joined together one by one (or at least as small peptides), and thus gradually built up into a large molecule. The intermediates may be peptides or simple derivatives of peptides. On the basis of the peptide theory, it is necessary to postulate a large number of specific enzymes in order to arrange the amino acids in their precise sequences.

Attempts have been made to demonstrate peptide formation under high pressure; the pressure must displace the equilibrium in the direction of peptide synthesis. However, when insulin from which the terminal alanine residue had been removed, was treated with labeled alanine in aqueous solution at pressures up to 6000 atmospheres, no intact insulin was formed. Nor was the terminal alanine of insulin exchanged for free radioalanine in the presence of carboxypeptidase[41].

Variations in the specific activity of the same amino acid in different parts of the protein molecule have been regarded as evidence for the existence of intermediate stages in protein synthesis, since they seem to indicate that the amino acid was not taken up all at once from the same pool. The experimental procedure is to produce a labeled protein by administration of a radioactive amino acid, to degrade it partially, isolate the different peptides, and hydrolyze them to their constituent amino acids. The specific activity of the same amino acid from each peptide is determined separately; if it varies from peptide to peptide it may be assumed either that this amino acid was incorporated into different parts of the protein by way of different intermediates, and that the pools of the intermediates were of different sizes, or that the amino acid was taken up from the same pool, but at different times, depending on the intermediate into which it was first incorporated. In either case dilution of the active by inactive substance might have occurred to different extents[265].

Non-uniform labeling has been observed in the case of ovalbumin synthesized *in vitro* in the presence of radioalanine from surviving hens' oviduct. A well-defined hexapeptide derived from the albumin exhibited far higher activity in the N-terminal alanine than in the C-terminal alanine[45]. Similar results were obtained on incubation of an oviduct mince with radioactive glycine[266]. The aspartic and glutamic acids in albumin synthesized from surviving oviduct or from mince also exhibited specific activities which varied with the position in the molecule[45, 266, 267]. Non-uniform labeling was found when pancreas tissue slices were incubated with glycine, and glycine and serine isolated from the resulting insulin[267], or when pancreas slices were incubated with phenylalanine, and the phenylalanine from the ribonuclease was examined[268]. Analogous results were obtained in the incorporation of glycine into hemoglobin[269]. Pronounced effects were noted in silk fibroin from worms injected with labeled glycine[270].

The results of other work were different; possibly the differences are due to the fact that the previous authors used tissue slices, minces, etc., rather than intact animals. Lactating goats were injected with labeled amino acids, and the amino acids were isolated either from the casein or from the lactoglobulin produced. The amino acids thus obtained always had the same specific activity; it was concluded, therefore, that they came from the same pool—*i.e.*, presumably from the amino acid pool. When the amino acids were isolated from the peptides obtained by partial degradation of the proteins, the same specific activity was again observed [53, 271]. When inactive lysine-containing peptides prepared from casein were injected simultaneously with radiolysine, the lysine isolated from the newly-formed casein still proved to be uniformly labeled, irrespective of its position in the casein molecule[272]. Uniform labeling has also been observed in other systems[273, 274]. Ferritin formed by rats in the presence of labeled amino acids (valine, leucine, isoleucine) contained amino acids with the same specific activity as the free precursors[97, 307]. Bence-Jones protein[276, 277] and the protein of pancreatic juice[278, 279] are also reported to be synthesized from the pool of free amino acids.

Attempts have been made to reconcile the experiments, which resulted in uniform labeling of the amino acid residues, with the hypothesis of free intermediates[265] (also called the transpeptidation hypothesis). On the other hand, non-uniform labeling appears to constitute evidence for the peptide hypothesis only if one assumes that the synthesis of every individual protein molecule on a template occurs rapidly; it is not known whether this is the case[280, 281]. Actually there is considerable evidence that the condensation on a template does not occur with very great speed[280, 281].

Certain experiments on tissue cultures of fibroblasts only seem to support the peptide theory. The proteins of chicken embryo extract, which was a component of the nutrient medium, were labeled by injecting a radioactive amino acid (phenylalanin) into the eggs before the incubation. An excess of unlabeled amino acid was added to the medium as a bank. Nevertheless, the newly-formed proteins in the tissue became strongly active. In this instance[141] as well as in others[282–285] it was concluded that fragments of moderate size (peptides) were released and later incorporated as such into the proteins. However, it is quite uncertain—even aside from objections against the analytical technique[97]—whether the inactive amino acid used to flood the pools actually gets to the site of the reaction in sufficient quantity.

A surprisingly large part of ^{35}S-methionine, which was released on degradation of serum albumin *in vivo* (rabbits, rats), was incorporated into serum globulin, and vice versa[108, 110]. Similarly, a re-incorporation of ^{14}-lysine from the serum protein of dogs into the tissue protein was observed; in this instance, the small extent of $^{14}CO_2$ formation was noteworthy[25]. It is apparent that in these cases the amino acid does not equilibrate with the pool; it may be presumed that the freshly-liberated amino acid is utilized preferentially for protein synthesis[152]. Also on the basis of experiments with doubly labeled proteins it has been argued that complete breakdown to the amino acid stage must precede re-incorporation[227].

Among the peptides occurring in mammals, glutathione has been most often studied[5, 286, 287]. Glutathione formation in liver homogenates has been investigated with labeled glycine. In this case, at least, the peptide is built up stepwise from the three amino acids. Two different enzymes and ATP (see below) take part[288–294] (see p. 166). Similar work has been done on the synthesis of glutathione by plants[295, 296]. The turnover rates of glutathione in several organs of the rat (erythrocytes[297, 298], liver[299, 300] and brain[299]) have been determined with labeled glutathione; from the observed constancy of the ratio of the specific activities of the three amino acids it was concluded that the peptide, once formed, does not exchange residues with free amino acids. In *Torula utilis* a half-life of 22 minutes has been found for glutathione under certain conditions[301].

From the standpoint of the peptide theory it would be best if the kinetics of the production of labeled peptides and labeled proteins were to fit the sequence amino acid → peptide → protein. Unfortunately, the isolation of peptides is not easy, and it is hard to know which peptides should be sought[263]. For these reasons, only a few studies of this sort have been carried out.

Turba has grown *Torula utilis* with labeled acetate as the sole source of carbon. The specific activities of the amino acids, peptides and proteins were determined separately at various times; approximately 40 different peptides were proved to be present, though in small concentrations, and were analyzed individually. All the common amino acids occur in these peptides. It was concluded that the peptides are closer in time to the proteins than are the amino acids. This result speaks in favor of the peptide hypothesis[301–304]. On the other hand, results indicating that labeled peptides are not utilized without being degraded first (glycylglycine[305]) are hardly significant, even if the 'right' peptide is used, because one can never be certain that the peptide actually reached the reaction site[306].

If the incorporation of an intermediate proceeds at a constant rate, the specific activity of the protein must also increase at a constant rate, once the intermediate pool has attained its maximum specific activity. Before this, the activity increases more slowly. (The kinetics are more complicated if the labeled amino acid must itself be formed first—for example, from labeled fructose[79].)

When labeled amino acids were incorporated into protein by chicken liver slices, it was found that labeled serum albumin (identified serologically and electrophoretically, and by its solubility) did indeed appear after 15–20 minutes only, but that a related labeled protein appeared earlier. From the time dependence of the radioactivities of the various fractions it was concluded that the latter protein was a precursor of the albumin [132, 309]. When ^{14}C-glycine or -leucine was injected into the veins of rabbits, activity appeared in the serum proteins after a 'transit' time of half an hour; in the next period the specific activity did actually increase linearly with time[310]. An induction phase has also been found in experiments on yeast[303]. These results can be explained either by the peptide theory or, if the formation and release of protein take place relatively slowly, by the template theory[281, 308].

An intermediate observed in protein synthesis with surviving hens' oviduct tissue has not been characterized chemically. Suspensions of oviduct tissue form protein from labeled amino acids. Homogenization of the tissue destroys this activity almost completely. On the other hand, if the suspension is first incubated with radioactive amino acids, and then washed and incubated with unlabeled cell sap, radioactive protein is obtained[311] (*cf.* Straub[197], p. 233).

Free energy is required to join amino acid residues by peptide bonds; the carboxyl group is activated by ATP[7, 134, 189, 312–315]. Enzyme systems have been found in tissue extracts from mammals[187, 189, 190, 316, 317], in microörganisms[318–321], and in plants[322, 323] which catalyze the exchange of radiophosphorus between ATP and inorganic pyrophosphate in the presence of amino acids[32, 324] (p. 163). Heavy oxygen is transferred to AMP from the carboxyl group of amino acids during their activation, as would be expected[1, 325]. The observation that the exchange between ATP and pyrophosphate is accelerated by the simultaneous introduction of several different amino acids is evidence that the enzymes are specific for the amino acids concerned. It will be recalled that the intermediates consist of amino acyl adenylates.

Subsequently it has been shown in a number of laboratories that the enzymes which catalyze the formation of amino acyl adenylates also catalyze the formation of amino acid-RNA compounds. The soluble acceptor RNA is only part of the total RNA of the cell and contains a terminal adenylic acid group. This accepts in the ester linkage activated amino acid groups from the activating enzymes[326–331]. Adenosine amino acid ester is released from the compound on digestion with ribonuclease[332]. The acceptor sites function independently of one another, and strong discrimination against unnatural amino acids is observed[307]. Finally, the amino acids are transferred, in the presence of GTP, to the ribonucleoprotein particles in the microsomes[332a] Clearly all these detailed results give strong support to the template theory.

The release of amino acids from proteins in living cells may likewise require a source of free energy. The release of labeled amino acids from rat liver or kidney incubated in buffer, in the presence of a bank of amino acid, was suppressed by the exclusion of oxygen or by dinitrophenol[247, 333,334]. The same is true of the degradation of adaptive enzymes in yeast [235]. A source of energy is also necessary for the induction of adaptive enzymes. In conclusion, we should like to refer to an interesting discussion of the template *vs.* peptide argument, in which Tarver points out that the questions have often been framed quite vaguely, and that various mechanisms are conceivable, which would combine features of both hypotheses[5].

6. The Metabolism of Amino Acids

A tremendous amount of work with labelled atoms has been done on the metabolism of amino acids[9,335]. Special reviews deal with the fates of carbon[336], nitrogen[337], sulfur[9,73,336, 336a] and aromatic amino acids[9,73,338] during the degradation and the transformations of the amino acids. The investigation of the mutual interconversions of amino acids with isotopes has been well supplemented and supported by work with defective mutants of microörganisms[339].

It was soon discovered that about 30 % of heavy nitrogen in glycine fed to normal rats was excreted in the urine within a short time, 20 % was incorporated into protein in the form of unchanged glycine, but 40 % was incorporated in other forms, and 10 % was converted to substances other than amino acids[3,14,340] (p. 226). Analogous results were obtained with other amino acids[14]. Labeled nitrogen was incorporated into protein even when the rat was fed a diet free of amino acids, and therefore was losing protein at a rapid rate[14, 17, 341].

The isotope content of the fed (physiological) amino acid is greater than that of any other amino acid in the protein[14, 22, 140]. On the other hand, the L-leucine within the protein is not especially active after feeding D-leucine; obviously the amino acid is deaminated, and the nitrogen redistributed[342–344].

Next to the amino acid fed, glutamic acid often has the highest content of heavy nitrogen. This amino acid plays a central role in transamination [73, 345–347]. After feeding labeled tyrosine or leucine, the ^{15}N content of the liver glutamic acid approaches that of the urea excreted. Since

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the nitrogen in the pool, which is available for redistribution, must have about the same isotope content as the urea nitrogen, no amino acid is expected to have a higher isotope content[14]. The mechanism of glutamic acid formation has been investigated with heavy nitrogen[344, 348].

The amino group of glutamic acid reacts rapidly. This fact has also been demonstrated with heavy water. Since deamination involves dehydrogenation, and amination involves hydrogenation, deuterium is incorporated into the amino acid in the course of the cycle. One day after the injection of heavy water into rats, all the amino acids except lysine contained deuterium bound to carbon. Glutamic acid again had the highest content of isotope; at least 80 % of the glutamic acid in liver had undergone the dehydration-hydration reaction[14].

These interconversions are not confined to amino groups; the carbon chains and the rings also participate[9, 14]. For instance, the conversions of ornithine (labeled with firmly-bound deuterium) into proline and arginine[349, 350], of labeled phenylalanine into tyrosine[351] and of isotopic serine into glycine (and vice versa)[9, 18, 73, 335, 336, 352] have been investigated. The amino acids non-essential for insects or mice were recognised by their radiocarbon content after rearing the animals on a diet containing radioglucose[353, 354].

On the basis of experiments with isotopes, Schoenheimer divided the essential amino acids into two groups. The amino groups of some of these amino acids, *e.g.*, leucine and histidine, contain biologically-labile nitrogen, which can be taken up and given off, but the carbon skeletons of these amino acids must be provided for the animal (rat).

In the case of lysine, not even the amino group can be derived from a precursor, and this amino acid must be supplied as such to the mammallian organism[14]; however, lysine is an example of an amino acid of which the metabolism follows different pathways in microörganism from those in higher animals[9].

Krebs and Henseleit have shown that in mammals the nitrogen of the amino acids is eventually converted into urea by way of citrulline and arginine. The mechanism has been confirmed with heavy nitrogen and radiocarbon[9, 14, 73, 355, 356]. The incorporation of carbon from CO_2 into urea was demonstrated with heavy and radioactive carbon[357, 358]. It is interesting that the urea nitrogen can be partially re-utilized by rats; the breakdown is mainly due to gastrointestinal bacteria[359, 360].

In conclusion, we may mention some studies of the production of various amino acids from other body constituents, and of their conversion into other metabolites[9, 73, 335, 361–363]. The formation of amino acids from glucose labeled in various positions in the mouse brain has been studied[364]. The reversible transformation of members of the citric acid cycle into amino acids (especially alanine, glutamic and aspartic acids) is, of course, of great significance[9, 345, 346, 365–367] (p. 197); thus, reactions of the citric acid cycle play a part in the incorporation of labeled carbon from CO_2 and other sources into amino acids by *E. coli*[368, 369]; the mechanisms of the rapid incorporation of radiocarbon from CO_2 into amino acids in *Pseudomonas* are different[370] (p. 179). The synthesis of radioactive amino acids from radioactive precursors by yeast has been investigated[371–373]. Work has also been done on the formation of aspartic and glutamic acids by green leaves during radiophotosynthesis[374–376] (p. 173).

Studies of the amino acids produced by *T. utilis* and by *E. coli*, grown with doubly-labeled acetic acid $^{13}CH_3{}^{14}COOH$ as the sole carbon source proved instructive; the isotopic carbon in the individual positions of the amino acids could be determined after selective degradation[377–379]. With labeled acetate or pyruvate as the only source of carbon similar methods were applied to *S. cerevisiae*[380].

The formation of fatty acids from glycine[9, 381], of acetoacetic acid from leucine, tyrosine and phenylalanine[9, 382, 383], and of adrenalin from phenylalanine and tyrosine[384, 385] have also been examined. The nitrogen of creatine comes partly from glycine, and partly from arginine[14].

The use of isotopes for the study of the mechanism of alkaloid formation shows good prospects for the future[386–389]. The synthesis of nicotine has been elucidated with labeled glycine, serine, lysine, tryptophane, ornithine, formaldehyde, and nicotinic acid[390–400]. Hyoscine (scopolamine) and hyoscyamine were also investigated[401, 402]. Labeled tryptophane, tyrosine and methionine were employed in work on the biosynthesis of methyltyramine, hordenine and gramine[403–406]; tryptophane was also used in studies of damascenine and trigonelline[407]. Other research was concerned with the origin of the N-methyl group of ephedrine[408] and the fate of the methyl group of nicotine[409].

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CHAPTER XV

THE PRINCIPAL PROBLEMS OF INTERMEDIARY METABOLISM, E

E. NUCLEIC ACIDS

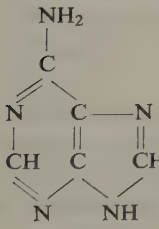
1. The Synthesis of Purines and Pyrimidines

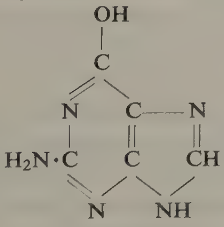
We shall first discuss the formation of the purine and pyrimidine bases, later the biosynthesis of their nucleosides and mononucleotides, and in the end take up the formation and reactions of the nucleic acids.

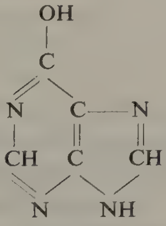
It is not known *a priori* whether the free purines and pyrimidines and their nucleosides and mononucleotides are produced by the same mechanisms as are the corresponding components of the polynucleotides (RNA and DNA). However, no qualitative differences in the reaction pathways have been discovered so far. Nor do the different forms of life appear to differ in principle. Therefore in order to economize on hypotheses, we shall start from the assumption that the reaction pathways are fundamentally similar.

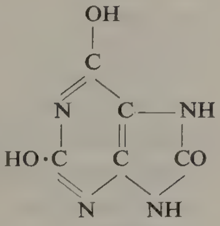
A comparison of the results of isotopic investigations with those obtained in genetic studies is very valuable. Many mutants of microörganisms are known which lack the enzymes for particular steps in the biosynthesis of nucleic acids[1,2].

In order to ascertain the origin of the individual carbon or nitrogen atoms of the purines, the system is provided with the supposed precursor in labeled form, and the purine bases of the nucleic acids (adenine and guanine) or their metabolites (*e.g.* hypoxanthine or uric acid) are isolated.


adenine


guanine


hypoxanthine


uric acid

The sites of labeled atoms are determined by suitable degradative procedures[3–7].

The first studies were carried out *in vivo*—on mammals, birds, or microorganisms—or with tissue slices. Later it proved possible to work with homogenates[8–12] and with cell fractions[11,13]. Even soluble extracts (of liver) can be used[4,14]. In cell-free systems dfficulties due to impermeability of the cell walls or to reactions on the walls do not arise.

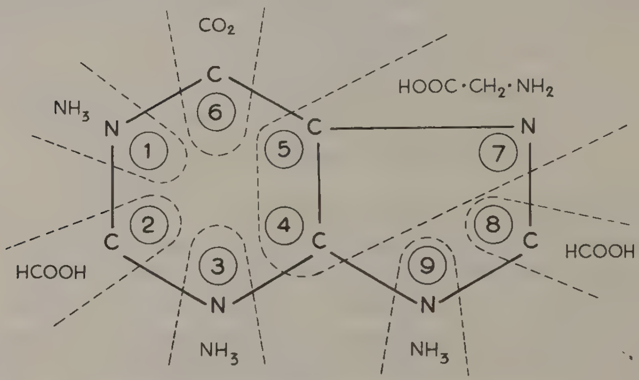
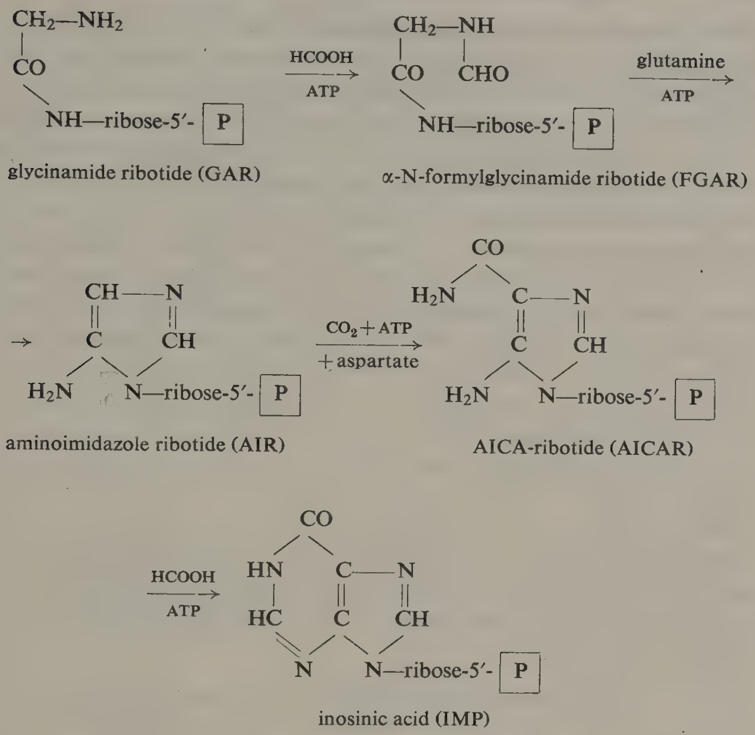


Fig. 26. Origin of the atoms of the purine skeleton.

The formula in Fig. 26 summarizes the results on purines[4]. It should be added that aspartic acid and glutamine have proved to be more active than ammonia; nitrogen atoms 3 and 9 are derived from the amide nitrogen of glutamine, but nitrogen atom 1 comes from the amino group of aspartic acid[6,7,15,16]. On the other hand, the nitrogen atom of urea is not incorporated into purines at all[17]. A remarkable parallelism has been found between the general mechanism of purine synthesis and—in the opposite direction—the fermentative degradation of purines by bacteria[18].

Work with liver has shown that carbon from labeled glycine, formate and CO_2 is incorporated into purines in the proportions 1 : 2 : 1, when all these substances are provided in ample quantities[6,14]. We assume that these proportions are characteristic of newly-synthesized purines, as is shown in Fig. 26. Nitrogen and carbon of doubly-labeled glycine ($^{15}NH_2 . ^{14}CH_2COOH$) are incorporated in the ratio 1 : 1; this is further evidence that the glycine molecule is used as such[4].

The ribotide of 4-amino-5-imidazolecarboxamide (AICA) is assigned an important place as an intermediate in purine synthesis[4,6,12,19]. Labeled AICA ribotide is utilized by vertebrates[9,20–22] and by yeast[23]. The AICA ribotide and later the purine derivative inosinic acid may arise from glycinamide ribotide through the reaction sequence:


HCOOH
ATP
glutamine
ATP
glycinamide ribotide (GAR)
α-N-formylglycinamide ribotide (FGAR)
CO₂ + ATP
+ aspartate
aminoimidazole ribotide (AIR)
AICA-ribotide (AICAR)
HCOOH
ATP
inosinic acid (IMP)

Formylation is effected by formyltetrahydrofolic acid (citrovorum factor). The intermediates in the formation of the glycinamide ribotide have also been determined with the aid of labeled atoms[12, 24–31].

The final step in purine synthesis is the formylation of AICA ribotide to inosinic acid (IMP, inosine monophosphate), the nucleotide of hypoxanthine[6, 32–37]. The inosinic acid serves as the starting point for the synthesis of the purine nucleotides which occur in nucleic acids[32, 33, 38]. (See however Carter[19]). Moreover, in the presence of the required enzymes the inosinic acid is in equilibrium with hypoxanthine and ribose-5-phosphate. The nucleotide is formed before the free base, so that radiocarbon made available as formate appears in the hypoxanthine later than in the inosinic acid[9, 32, 33, 39–41].

The inosinic acid yields—in extracts of *E. coli*[42] and of bone marrow[43]—adenylic acid. The mechanism of the transformation is as follows: the reaction with aspartate in the presence of GTP (guanosine triphosphate) first yields succinyladenosine-5′-phosphate (substitution of the amino nitrogen of adenylic acid). Thereafter, fumarate is split off, as was demonstrated earlier with yeast[44]. Experiments with heavy oxygen have indicated the probability that 6-phosphorylinosinic acid is formed first, and that subsequent replacement of the phosphate group by aspartate yields the succinyladenosine-5′-phosphate[42, 45]. Alternatively, guanylic acid is formed when the inosinic acid is first oxidized to xanthosine-5′-phosphate (xanthine = 2,6-dioxypurine), and this XMP is aminated in the presence of ATP; ammonia acts as an amino group donor in extracts of *Aerobacter aerogenes*[46], and glutamine, in bone-marrow extracts[43], in HeLa cell cultures[16] or in pigeon liver extracts[47]. The conversion of nucleic acid adenine to nucleic acid guanine also proceeds by way of IMP and XMP[46, 48]. These reactions of inosinic acid are discussed in review articles[2, 19].

Metabolically, the amino group of guanine is closely connected with the nitrogen pool of the organism. When $^{15}NH_4Cl$ is administered to rats, the heavy nitrogen appears in this amino group earlier than in the ring nitrogen. Isotopic ammonium chloride also labels the amino group of adenine relatively quickly, though not as rapidly as that of guanine[4, 16, 17, 38, 49, 50].

We have explained how ribotide formation can occur before the completion of the purine ring. Enzyme systems have nevertheless been obtained from liver and yeast which are capable of bringing about reversible reactions of the type:

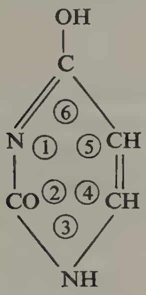
adenine + 5-phosphoribosyl-1-pyrophosphate (PRPP) $\rightleftharpoons$ AMP + pyrophosphate (PP)

(p. 163) or the analogous reactions with guanine, hypoxanthine, or AICA. The pathway from AMP to ADP and ATP is well-known. At least in yeast, nucleoside triphosphates of other bases are formed from the corresponding monophosphates by transphosphorylation with ATP. Transphosphorylation from polyphosphate to ADP, with formation of ATP, has also been established[51, 52]. In these ways, the free bases, and even free AICA, can be made available for the formation of various nucleotides, and hence, of nucleic acids[53–57] (see below).

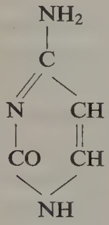
Under certain experimental conditions, radiocarbon from formate is incorporated into inosinic acid in much larger quantities than would correspond to the *de novo* synthesis of inosinic acid, as measured with ^{15}N-glycine. For example, in the presence of an excess of unlabeled inosinic acid, or in the absence of bicarbonate, *i.e.* in conditions which depress the *de novo* formation of purines, instead of a ratio of 2 : 1 between the incorporation of ^{14}C and of ^{15}N, a ratio of 100 : 1 or more is observed. It is apparent that an enzymatically-catalyzed exchange occurs between the formyl carbon and the carbon in the 2-position[4, 9, 34, 37, 58, 59].

Studies on rats[60, 61] and mice[62] have definitely shown that the exchange of atoms 2 and 8 is not important *in vivo*. No matter which carbon atom (2, 4, 6 or 8) of the adenine was labeled, the same specific activity was obtained in the nucleic acid. The ratio of labeled carbon (position 8) to that of heavy nitrogen (positions 1 and 3) also remained constant during the incorporation of the adenine. The ring system of adenine may therefore be regarded as stable in the living animal.

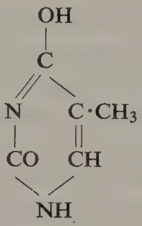
The biosynthesis of pyrimidines (uracil, cytosine, thymine) was investigated after that of the purines, and it was found that the pathways are entirely different[38, 63, 64]. Selective degradation methods to determine the location of the isotopes have also been worked out for the pyrimidines [38, 65, 66]. Neither the nitrogen nor the carbon of glycine[67–69] nor the carbon of formate[67, 69] are utilized for pyrimidine synthesis. Pyrimidines are not natural precursors of purines[38, 70, 71].



uracil



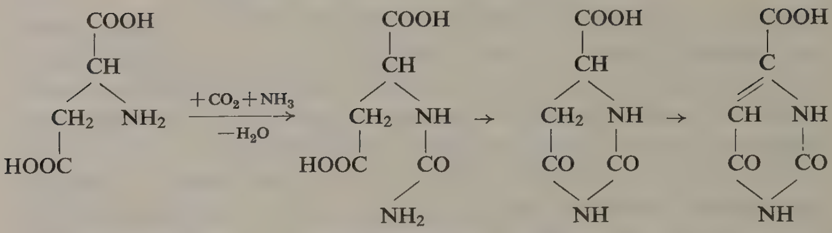
cytosine



thymine

An important intermediate in the biosynthesis of pyrimidines is orotic acid (uracil-4-carboxylic acid). Labeled orotic acid gives rise to labeled pyrimidines (cytosine and uracil derivatives) in the RNA and DNA of rats[38, 72–78], of rat liver slices[38, 72–74, 77] and homogenates[11], of tumor tissue[79], and of yeast[69]. The nucleotide of orotic acid, orotidine-5′-phosphate (orotidylic acid) is formed by reaction with PRPP, and yields uridylic acid on decarboxylation[80–83]. However, enzyme systems occur (*e.g.*, in the cytoplasm of rat liver[84,85] or in microörganisms[86]) which convert free uracil into uridylic acid. Lieberman has elucidated the formation of cytidine nucleotides from uridine nucleotides by *E. coli*[87, 88]. It has been shown by the isotope-competition technique[88] that the ring of thymine can be derived from that of uracil[38, 72–74, 89].

Orotic acid is formed from ureidosuccinic acid (carbamylaspartic acid). The latter, in turn, is built up from aspartic acid, CO_2 and NH_3 in the presence of ATP. Enzymes effecting these conversions have been obtained from rat liver and from *E. coli*, for example. The details of the synthesis of orotic acid are still being debated[19, 38, 90–95].



aspartic acid ureidosuccinic acid dihydroorotic acid orotic acid

The methyl carbon of thymine may arise from labile methyl groups or from formate[67, 68, 73, 89, 96–103]. 5-Hydroxymethyldeoxycytidylic acid, which occurs in certain phages, is produced enzymatically in the presence of tetrahydrofolic acid from deoxycytidylic acid and (radioactive) formaldehyde[101].

It has been observed that even very small quantities of uracil or cytosine (but not thymine) inhibit *de novo* synthesis of ureidosuccinic acid, and hence, of orotic acid, in *E. coli*. This effect has been ascribed to the existence of a mechanism which adjusts the production of pyrimidines to their incorporation into nucleic acids, and it has been further postulated that the inhibition of the formation of intermediates by the end products of a synthetic pathway is a general regulatory mechanism in all cells and has the effect of directing metabolism into the required channels[104]. Accordingly, the rates of the reactions are controlled by the quantity of end products[105, 106].

The degradation of purines and pyrimidines under physiological conditions can, of course, be studied very well with the aid of isotopes[4, 9, 64, 92, 107–112]. For the synthesis and catabolism of ribose and deoxyribose, see Sections *XI*, *3* and *XII*, *2*, and the literature[113–121].

2. The Role of Free Bases, Nucleosides, and Nucleotides in the Synthesis of Nucleic Acids

Experiments with labeled atoms demonstrate, as was shown in the last section, that the biosynthesis of polynucleotides involves not only such simple precursors as formic acid and glycine, but also such preformed intermediates as the bases, nucleosides and nucleotides. The biosynthesis[5, 122] and the reactions[123] of the nucleosides and nucleotides have been discussed in recent reviews (*cf.* also section *1*). We now propose to survey the role played in biosynthesis by bases, nucleosides and nucleotides.

Most of the work undertaken to decide the question whether the free bases (adenine, guanine, uracil, cytosine, and thymine) or the bases present as nucleosides or nucleotides are incorporated into nucleic acids has been carried out on rats[63, 64]. Occasionally, tumor-bearing rats have been studied[79, 124, 125]. Some other organisms—including purineless mutants[1] of *Aerobacter*[48] and protozoa[126]—have been also used. Cell-free systems also incorporate purines and pyrimidines into nucleic acids (see below).

The rat can introduce free adenine into the nucleic acids of the liver with retention of the purine skeleton, either as adenine itself, or after transformation into guanine[108,127, 128]. Mice incorporate free adenine into RNA; the speed of the process varies greatly, depending on the organ involved[129, 130]. The adenine and guanine of both the RNA and DNA of fibroblast tissue cultures become radioactive on incubation with ^{14}C-adenine[131] (p. 266). Free guanine is incorporated into liver[117,127, 128] and fibroblasts[131] to a smaller extent than is adenine. Different microörganisms have different capacities for the mutual conversion of adenine and guanine during their incorporation into polynucleotides[4,63, 117, 132].

It appeared at first that—in contrast to orotic acid—the free pyrimidines uracil[70], thymine[70], and cytosine[71] could not be used at all for the synthesis of nucleic acids in the rat. Later, however, it was found that uracil could be used quite well provided rapid degradation of the base was prevented[79, 84, 112, 133].

Not only free adenine, but also the adenine moieties of nucleosides and nucleotides are used for nucleic acid synthesis[63, 117]. In this process the three isomeric adenylic acids (and also the *a*- and *b*-isomers of guanylic acid) behave similarly[134, 135]. The nitrogen-labeled pyrimidine moieties of the nucleosides cytidine and uridine are also incorporated into RNA and DNA[136–138]. During nucleic acid synthesis, the purines present as nucleosides are partially converted into one another; the same holds true during RNA synthesis for the pyrimidines uracil and cytosine[73, 136–140]. Simultaneous methylation to thymine (see below) takes place when labeled uracil, present as a deoxynucleoside, is incorporated into DNA.

Brown (Table 11) has compiled the data obtained by various workers on the incorporation of purines, pyrimidines, nucleosides and nucleotides

TABLE 11

INCORPORATION OF BASES, PRESENT AS FREE BASES, NUCLEOSIDES, OR NUCLEOTIDES, INTO THE RIBONUCLEIC ACID OF RAT LIVER

	Free Base	*Nucleoside*	*Nucleotide*
Adenine	++	+	+
Guanine	+−	+−	+
Uracil	+−	+	+
Cytosine	−	++	++

into rat liver RNA[63, 141, 142]. The significance of the data is not affected by the fact that only the 2′ and 3′-isomers of the nucleotides are included (see below). The data refer only to the incorporation of the labeled bases themselves, even though they may be made available as nucleosides or nucleotides.

Special isotope experiments were required to decide the further question whether or not the ribose residue was incorporated together with the base by various organisms. For this purpose both components of the nucleoside were labeled. When cytidine with radiocarbon in both the ribose and the base was used, the activities of the cytosine and sugar incorporated into the RNA were in the same ratio as those in the cytidine employed. When cytidine was isolated from the nucleic acid, the specific activities of the two components were again in the same proportion. It is evident that the glycosidic linkage is preserved during the incorporation. The same result was found with uridine[138, 143–145]. The linkage was also maintained when adenosine, administered to rats in the form of adenylic acid, was converted to guanosine and incorporated into polynucleotides[144]. Indeed, the nucleoside bond can even be preserved when the ribose is reduced to deoxyribose, and the nucleoside incorporated into DNA[73, 138, 143–146]. In *E. coli* infected with T_1-bacteriophage, labeled uridine was converted into DNA-thymidine with maintenance of the nucleoside bond[147]. In chick embryo, the synthesis of deoxyribose from ribose takes place at the nucleotide level[148].

On the other hand, uracil deoxyribonucleoside is not utilized for RNA synthesis in the rat; the labeled base (after methylation) turns up only in the DNA. Apparently the deoxyribose cannot be converted to ribose[73, 146, 149]. ^{14}C-thymidine is incorporated into DNA (but not into RNA) by many mammalian[73, 146, 149, 150] and bird[149, 151] tissues; no incorporation of the carbon into other portions of the DNA molecule was observed.

Labeled precursors, which are used exclusively for the synthesis of DNA (and not of RNA), are of course of considerable scientific interest. Thus ^{3}H-thymidine is used to an increasing extent in important experiments, in several organisms, on the distribution of parental DNA among the progeny *i.e.* on the mechanism of replication of DNA; the technique of autoradiography is of utmost importance in these studies[152–163].

We turn from the nucleosides to the nucleotides. Many experiments on the incorporation of nucleotide phosphorus have been performed. For example, when any ^{32}P-nucleotide was administered to rats, and the nucleic acids of the various organs degraded to mononucleotides, radiophosphorus appeared in every fraction. It was concluded that the phosphate is largely split off and redistributed during nucleic acid synthesis[164]. This is in accord with the finding in experiments on animals, tissue slices and cell suspensions that no difference can be observed in the utilization of the phosphorus from 2′-, 3′- or 5′-AMP; the phosphate is split off and mixes with the pool of orthophosphate[150, 165]. This picture has been confirmed *in vivo* by the use of triply-labeled nucleotides (^{15}N-base, ^{14}C-ribose and ^{32}P-phosphate); during the incorporation of the bases and nucleosides the phosphate came into equilibrium with the pool of free phosphate[144].

When guanylic acid is administered, the guanine incorporated into the nucleic acid is accompanied to only a slight degree by the ribose of the nucleotide, and not at all by the phosphate. Guanine in guanylic acid is nevertheless much better utilized than is free guanine; the presence of the phosphoribosyl group seems to improve the uptake of the base, although the mechanism is not known[144, 164, 166]. In the case of adenine, no such effect appears; the uptake of the bound base is less than that of the free base.

The phosphorus seems to be split off during the passage through the cell wall[165]. For this reason quite different results are often obtained in cell-free systems and in tissues. Adenine in the form of AMP is utilized by cell-free liver homogenates even better than free adenine; the ribose, and to a large extent even the phosphate, remain attached[167]. When the nucleic acid of rat liver homogenate incubated with radioactive AMP was hydrolyzed enzymatically, the highest specific activity of the phosphate was observed in the AMP[10] (see also[168]).

On the other hand, when ^{32}P-AMP was incubated with liver homogenate, and the RNA hydrolyzed with alkali, much *less* activity was found in the AMP than in the CMP, UMP or GMP. According to our present concepts, alkaline hydrolysis yields nucleotides in which the phosphorus originally in the 5′-position of one nucleotide is esterified to the 3′-position of the adjacent nucleoside. For this reason, the very small amount of activity of the AMP indicates that the phosphorus was not first split off as inorganic phosphate. It also proves that in these nucleic acids the AMP usually adjoins nucleotides other than AMP. If either one of these two assumptions were untrue, the radiophosphorus would have to be distributed more evenly among the mononucleotides[167]. When *E. coli* was grown in a medium containing thymine, and later transferred to one containing both 5-bromouracil (which replaces the otherwise essential thymine) and $^{32}PO_4$ ions, a particularly high radioactivity was found in those nucleotide moieties of the DNA which contained the bromouracil; this result also indicates that nucleic acids are formed from nucleotides[169].

The weight of the evidence obtained in these and similar experiments is that the biosynthesis of nucleic acids proceeds more readily from nucleotides than from free bases or from nucleosides. Accordingly, the bases and nucleosides are first phosphorylated. This happens inside the cell.

Enzymes which catalyze the formation of nucleic acids from nucleotides have been discovered recently. Only a few examples will be given here.

The reversible synthesis of RNA-like polynucleotides ('synthetic nucleic acids') from diphosphoribosyl purines or pyrimidines, according to the reaction

$$n(\mathrm{X{-}R{-}P{-}P}) \rightleftharpoons (\mathrm{X{-}R{-}P})_n + n\mathrm{P}$$

(X = adenine, hypoxanthine, guanine, uracil, etc., R = ribose, P = phosphate), in extracts of *Azotobacter vinelandii*[170–172] or *E. coli*[173] can be followed radiochemically by introducing ^{32}P-XRPP and measuring the release of labeled inorganic phosphate. The extract also catalyzes an exchange of the terminal phosphorus of the diphosphate with inorganic phosphate even after equilibrium has been reached; but inorganic phosphate does not, of course, exchange with the mono- or triphosphonucleotides. Oligonucleotides are required to 'prime' the synthesis of polynucleotides[174, 175]. The phosphorolysis of various ribonucleic acids, *i.e.* the reverse of synthesis, takes place at different rates[176]. The incorporation of nucleotides into RNA-like polynucleotides has also been followed in extracts from *M. lysodeikticus*[177]. Moreover, copolymers containing different bases can be produced by reactions of this type. It was shown with radiophosphorus that the bases may be randomly distributed in the polynucleotides[178]; the sequence then may not convey 'information'.

More recently, the uptake of labeled nucleotides into RNA-like material by soluble extracts from mammalian cells, *e.g.*, from ascites tumor cells[179] and from rat liver[180, 181], has been reported. However, no net synthesis has been proved so far[181], and the relationship of these phenomena to those connected with protein synthesis (p. 240) is not clear (see, in addition[182, 183]).

The enzymatic synthesis of polynucleotides resembling DNA in extracts of *E. coli* has also been studied with radiophosphorus. The reaction occurs only when the four triphosphates of thymidine, deoxyadenosine, deoxyguanosine and deoxycytidine, along with a DNA primer, are present simultaneously. Inorganic pyrophosphate is released in quantities equivalent to the number of nucleotides incorporated[184–187]. The incorporation of ^{14}C-deoxynucleotides into DNA in soluble fractions from vertebrate tissue has been demonstrated[188–190].

Many authors have reported attempts to incorporate analogues of bases, nucleosides, and nucleotides, or to inhibit growth by the application of such antagonists[191–214].

3. The Synthesis and the Dynamic State of Nucleic Acids

In the preceding sections the reaction sequences in the biosynthesis of nucleic acids have been discussed. We turn now to the investigation of the rate of renewal of nucleic acids with isotopes, *i.e.* to the problem of the dynamic state. The interpretation of the experimental results is just as difficult as in the case of proteins.

Hevesy employed radioactive phosphate to measure the turnover of nucleic acids[215, 216]. Since the nucleic acid may adsorb some highly radioactive inorganic phosphate even after isolation, the best analytical procedure consists in degrading the nucleic acid (DNA or RNA) to the four mononucleotides, separating them by electrophoresis, and assaying them individually for their activities[64, 217, 218]. Later work was carried out with labeled purines and other organic components or precursors of nucleic acid. Work with highly-active precursors can be avoided by labeling *in vivo* and following the decline in activity (*i.e.*, the 'retention') instead of the increase in activity. The results of many determinations of turnover rates have been compiled[63].

We first present a few general results, most of which were obtained with liver tissue. The livers of adult rats, mice and rabbits[215, 216, 219–223] incorporate radiophosphorus into DNA only slowly, as do other mammalian tissues which are not in an active state of growth[224, 225]. (The incorporation—in various kinds of tissues—strongly depends on the mitotic phase [226, 227]). On the other hand, phosphorus is usually incorporated rapidly into RNA[215, 216]. In one experiment, for example, the specific activity of the total RNA of rat liver was 33 times greater than that of the DNA two hours after the administration of labeled phosphate[220].

The ribonucleic acids of the various components of the cell, which differ in their chemical compositions[230, 231], also incorporate isotope at different rates. The turnover is most rapid in the RNA of the nucleus[215, 216, 220, 232–239]. Cytoplasmic RNA is more active than nuclear DNA, but less active than nuclear RNA; differences exist between cell-sap and subcellular particles (mitochondria, microsomes)[64, 221–223, 231, 237–240] and even between different fractions of the microsomes[241]. Preferential incorporation of radiophosphorus into the nuclear RNA has also been found in the unicellular giant alga *Acetabularia mediterranea*[234–236, 240] (see p. 234, 267) and in *Drosophila*[233, 242]. The metabolism of the cytoplasmic DNA in frog ovarian eggs is pronounced[243].

Similar results were obtained, in general, with ^{15}N-adenine, ^{15}N-glycine, ^{14}C-glycine and ^{14}C-formate; a rapid incorporation into the purines of rat liver RNA—especially in the nucleus—was observed, and the purines of the liver DNA also took up the isotope[64, 96, 98, 244–250]. Experiments with orotic acid, where pyrimidine metabolism was measured, again revealed that the RNA of the nucleus turns over more rapidly than that of the cytoplasm[78]. By autoradiographic examination, an especially active turnover was found in the nucleolus of the oocytes of starfish and frogs[251,252]. (See also *A. mediterranea*, p. 265).

Regenerating liver incorporates phosphorus and adenine into RNA (and DNA) faster than does normal liver[244,250,253–255]. The rates of incorporation into the liver of growing rats[256] and chicken embryos[149] are also high. Organs distinguished by high mitotic activities, *e.g.* bone marrow, spleen, and intestinal mucosa, incorporate rapidly into RNA and DNA even in the normal state[64,99,130,215,216,223,257,258]. In the case of brain, the dependence of the turnover of nucleic acid phosphorus on the phylogenetic antiquity of the part of the organ, and on the physiological condition, have been investigated[259,260]. The incorporation of phosphorus into the RNA of yeast decreases sharply on passing to the resting state[261]. Quite generally, the turnover of RNA is easily influenced by a number of factors[183,262,263]. We have already referred to the strong influence of ionizing radiation on the metabolism of the nucleic acids (p. 59). The synthesis of nucleic acids—as measured with phosphorus, formate, glycine, and other precursors—is greatly elevated in cancer tissue, although it falls off when necrosis sets in[219,222,248,264–269]. Some workers have found that guanine is incorporated into cancer tissue at an abnormally slow rate[125,269–272]. Autoradiography is a useful tool in the comparison of the uptake of labeled precursors *in vivo* by different tissues; descrimination between RNA and DNA is possible, *e.g.*, by treatment with ribonuclease before radiography[273,274] (see p. 87).

After preliminary investigations of nucleotide metabolism in tissue cultures[275], labeled nucleic acids (obtained by injection of ^{14}C-adenine into fertilized eggs) were employed to study the transfer of bound purines from the culture medium to the tissue. After addition of DNA or RNA, both containing radioactive adenine and guanine, to the chicken embryo extract serving as a culture medium for chicken heart fibroblasts, the DNA and RNA produced in the tissue were found to be similarly labeled in both purines. The conclusion was drawn that the products of the hydrolysis were re-utilized for the synthesis of fresh nucleic acids[131]. On the other hand, experiments on pregnant or parabiotic rats and on tumor-bearing mice revealed that the degradation products of nucleic acids labeled *in vivo* were not appreciably used for the synthesis of new nucleic acid once they had entered the general circulation[276].

Incorporation studies have been extended to isolated cell components. Hog kidney nuclei incorporate phosphate into RNA more rapidly than into DNA[277,278]. When the alga *Acetabularia mediterranea*, which has already been mentioned (p. 265), is cut into two parts, even the portion without a nucleus remains viable for several months; this may be due to the large number of chloroplasts it contains. If this portion is provided with labeled orotic acid or labeled phosphate at various times after the cell had been cut, not only a continued renewal, but an actual *de novo* synthesis of cytoplasmic RNA (and also a *de novo* synthesis of protein; p. 234) is observed[183, 279–284]. To a much slighter extent this also happens in *Amoeba proteus*; although the enucleate portion continues to live for weeks, it loses most of its synthetic powers[285, 286].

Much interest is shown in the question whether nuclear RNA is a precursor of cytoplasmic RNA, as has been suggested by Jeener and Szafarz [64, 231, 239]. New evidence for a genetic connection between the two kinds of nucleic acid, in the case of *Amoeba proteus*, has been furnished by autoradiography. Cell nuclei containing ^{32}P-RNA were grafted into enucleated cells, and the appearance of radioactive RNA in the cytoplasm was observed directly[287]. The opposite interpretation has been put on results obtained with *A. mediterranea*[236].

Brachet summarizes the situation as follows' Whether nuclear RNA is a direct precursor of cytoplasmic RNA cannot yet be decided with certainty. Although many facts are compatible with such a hypothesis, it is highly probable that, if such a transfer of nuclear RNA to the cytoplasm occurs, it is a more complex phenomenon than simple diffusion. Furthermore, it is very likely that nuclear RNA is not the sole precursor of cytoplasmic RNA'[282] (see also [183, 283]).

As early as 1941 Brachet in Belgium and Caspersson in Sweden speculated that RNA might be the essential constituent of the template on which proteins are synthesized. In Chapter XIV we have referred to the relationship between RNA contents of subcellular structures and the capacity for protein synthesis. Experiments with radioelements on the role of RNA in the activation of the amino acids have also been mentioned there. Now a number of reviews of work with isotopes to elucidate the role of RNA in protein synthesis may be cited[183, 210, 211, 282, 283, 288–290]. Certainly experiments on many systems show a strong correlation between RNA content and protein synthesis[291, 292]. The formation of adaptive enzymes, for example, is accompanied by an increase in RNA[293]. The synthesis of protein from labeled amino acids is inhibited by ribonuclease [183, 211, 231, 282, 283, 288–290], but there is no clear correlation between the turnover of protein and that of RNA[241]. The turnover of RNA continues in phage-infected bacteria though the amount of RNA remains constant [294].

DNA cannot be directly necessary for protein synthesis in all cases, despite the fact that the turnover of the proteins in the cell nuclei is rapid

[183, 283]. This is clear from the experiments with enucleated cells, which are, of course, free of DNA, and also from experiments on reticulocytes, which have no nuclei. These cells synthesize hemoglobin[295] and enzymes[296] and incorporate glycine into RNA[297]. (The correlation between the decrease in RNA content and the decrease in the rate of incorporation of glycine into reticulocytes has been directly demonstrated by autoradiography[298].) Experiments on the metabolism of amphibian eggs[299, 300] have also been interpreted as indicating a continuous turnover of RNA and protein without any help from the nucleus[183, 283]. We cannot assert, however, that DNA does not in the long run control the synthesis of protein, acting through RNA[183, 283].

Variations of the specific activity within the molecule make it more difficult to decide whether the various kinds of nucleic acids are in a dynamic state. After labeled phosphate had been given to mice the various mononucleotides obtained by enzymatic hydrolysis of the liver DNA contained different amounts of ^{32}P; thymidylic acid was most active[301]. The nucleotides of rat liver RNA, after administration of ^{32}P or ^{14}C *in vivo*, differed in activity[272, 302–309].

Not only are the activities of the different nucleotides of the nucleic acids different, but it has also been shown (with labeled phosphate and orotic acid) that the activity of one single nucleotide may differ, depending upon whether it occupies an internal or a terminal position in the nucleic acid molecule[272]. In this connection, it is well to recall that different parts of the molecules differ in their compositions with respect to bases, and in their vulnerability to enzymatic attack. This has been demonstrated in the cases of yeast and liver RNA[272, 310] and of yeast and thymus DNA[311].

These results make it appear likely that different quantities of isotope can be taken up by different parts of the nucleic acid molecules ('intramolecularly') in these preparations. The possibility must be kept in mind, however, that the RNA and DNA from the same site in the cell consist of different types of macromolecules which behave differently. This sort of ('intermolecular') variability from one molecule to another has been indicated by chemical analyses of the DNA of calf thymus[312–318], human leucocytes[314] and microörganisms[314, 316]. It has long been known that whole cells, for instance of yeast[319], contain various types of RNA[231, 317, 318], but the RNA from one and the same part of the cell (*e.g.*, the nuclei of calf thymus) has also been shown to be heterogeneous[320–322].

Different rates of labeling have actually been demonstrated (with radioformate) in different DNA fractions from various organs of the rat[323], with radiophosphorus in different DNA fractions from mouse liver[324] (however see also[325]), and in DNA from the bone marrow of chickens[326].

Two RNA fractions from the nuclei of calf thymus[327] and various RNA fractions from the nuclei of mammalian livers[328–330] or from starfish oocytes[331] also exhibit different rates of uptake and release of isotopes.

These differences in velocities do not lead to the direct conclusion that different turnover rates prevail. If the labeled material is administered all at once, as is usually the case, the possibility is not excluded that the pools of the various immediate precursors of the nucleic acids may have different specific activities. This may be due to differences in the sizes of the pools, or in the rates of transport of the precursors to the reaction sites (*i.e.*, differences in the rates of mixing of the labeled material with the pool), or both[63, 332]. Extensive kinetic studies of the purine pools in yeast have been undertaken[333].

A comparison of the rates of incorporation of different labeled precursors into the DNA of a single organ is of particular interest. In order to eliminate the effect of pool size, the ratio of the rates of incorporation of the two precursors into the RNA of the same organ has been used as an 'internal standard'; in other words, the assumption is made that the DNA and RNA draw on the same pool. The remarkable result was obtained that the ratio (q_1) of the rates of incorporation of adenine and of 'simple precursors' (glycine, formate) into the DNA of liver was much smaller than the ratio of the incorporation rates into liver RNA (q_2). Similar results were obtained under other experimental conditions; the value of the quotient $Q = q_1/q_2$ depends upon the organ involved, and upon other circumstances[4, 63, 64, 99, 128, 246]. The deviation of the value of Q from unity is especially apparent when adenine and simple precursors, labeled in different atoms, are introduced simultaneously[99, 128, 246]. Obviously various reaction pathways compete. This is also evident from the fact that the incorporation of isotope from glycine or formate into DNA is inhibited by adenine (see above).

The incorporation ratio, ^{14}C-adenine/^{15}N-glycine, into DNA (relative to the ratio for RNA, *i.e.*, the Q-value), was much higher in regenerating liver than in normal liver; exogenous adenine appears to play a more important role in DNA synthesis during growth[246]. It has been suggested that adenine can be taken up by nuclei only during cell division[128], but other possibilities of explanation exist[246]. Whatever the reasons for the differences, they must be taken into account in estimating turnover rates[4, 63, 64, 99, 128, 301].

Another source of error in the determination of turnover rates is—as in the case of proteins—the re-incorporation of degradation products; this source of error is not obviated when the turnover is measured by the 'retention method'. Its importance became evident in some studies on mammalian tissue cultures, which contained thymine-labeled DNA; the tissue exhibited a lowered retention when the nutrient solution contained a 'bank' of unlabeled thymine[334, 335]. Even in this case, however, there was no guarantee that re-incorporation was entirely eliminated, since the labeled degradation products are formed within the cells, and may be utilized in preference to the 'bank'.

In view of these shortcomings of the experimental techniques, it may be concluded only that different molecules, or different parts of the same molecules, are labeled at different rates; but it has not been proved that the different parts of homogeneous nucleic acid fractions (and hence, of single nucleic acid molecules) are renewed at different rates.

Further progress may be possible through the technique of 'continuous administration' which has not been employed very much so far, and which does not, indeed, give absolute protection against re-incorporation. If labeled material is provided at a constant rate, all the pools involved must approach uniform specific activity. From experiments with $^{14}CO_2$, Swick and his coworkers have concluded that all the RNA of young rats is renewed at the same rate. Later experiments with radiophosphorus confirmed these results, and afforded evidence that molecules of RNA and DNA are synthesized as complete units[336, 337].

These authors have also come to some conclusions about the dynamic state of liver DNA. Since the increase in DNA, and hence in DNA carbon, during the course of the experiments was known, and since the radioactivity of this carbon was measured at the end of the experiment, the specific activity of the 'new' DNA carbon could be calculated. The specific activity thus determined was the same as that of the precursor, and it was concluded that the newly-formed DNA, but not that previously present, had taken up carbon. Each mitosis, therefore, involves the synthesis of a unit of DNA, while the DNA already present remains unchanged.

More evidence against a dynamic state of the DNA has been obtained in experiments with ^{32}P on the liver of young rats[338, 339], on regenerating rat liver[253, 340, 341], on cultures of subcutaneous mouse tissue (Earle's 'strain L')[342, 343], on *Tradescantia*[344] and especially (with ^{32}P and ^{14}C) on growing *E. coli*[345–347].

There is, nevertheless, much to be said in favor of a dynamic state not only of RNA (where it is often very noticeable), but also of DNA. In systems of high mitotic activity, the dynamic renewal might take place, but be obscured by synthesis. Similar difficulties have arisen in the case of proteins (p. 235). A clear example of active metabolism of DNA in a non-dividing nucleus is the frog ovarian egg[348]. Another example is the seminal vesicle of the mouse[349].

The experiments with isotopes on the question of the dynamic state of the DNA have been carefully considered in a review by Mirsky and his colleagues[231]. Referring to certain experiments on the retention of radiocarbon by liver DNA[323], the authors remark: 'The results of isotope retention experiments are not compatible with the viewpoint that the DNA of a non-dividing cell is metabolically inert. The evidence points instead to a complex system in which some of the bases of the DNA may participate in exchange or renewal reactions . . . The variable utilization of labeled precursors for DNA synthesis is itself a matter of considerable biological interest for it implies that the chromosome is responsive to its environment.' Here reference is made to the factors affecting the relative rates of incorporation of different precursors into DNA and to the dependence of the absolute rates upon physiological conditions.

Examples of physiological factors affecting the incorporation of isotopes into tissues not actively dividing are known. Thus the uptake of glycine carbon by pancreatic DNA is increased when the gland is stimulated (by feeding) to produce enzymes[350]. The *in vivo* incorporation of formate carbon into rat liver DNA is changed when the animal is given N-methylformamide[351]. The rates of incorporation of orotic acid into the DNA of various mitotically inactive organs of an animal also depend upon the diet[231]. The authors conclude that 'neither the protein nor the DNA of the chromosome is inert in non-dividing cells. On the contrary, the picture which emerges is one in which the components of the chromosome are in constant equilibrium with their environment, altering their metabolic patterns in response to changes in the cytoplasm, and intimately related to, and possibly directing, many of the functional activities of the cell'.

As for the above-mentioned experiments with *E.coli*, 'a fundamental metabolic distinction may exist between the bacterial cell which is adapted for rapid growth and multiplies without 'turnover', and those mammalian cells in which 'turnover' is the reflection of the cell's capacity to adapt its composition to meet changes in its environment. It should be emphasized, however, that a state of 'dynamic equilibrium' is not a characteristic of all cells in higher organisms. The hemoglobin of the mature erythrocyte, for example, is not 'turning over'; it is laid down in the primitive red cell and remains for the limited life-time of the cell. Such an absence of turnover may be a general characteristic of tissues in which the cells themselves are continuously being replaced.'

The DNA of erythrocytes, like their protein, seems not to be in a dynamic state. Apart from radioactive decay, the activity of the DNA in the red blood cells of birds remains constant after biosynthetic introduction of radiophosphorus. Methods of determining the life span of the cells are based on this fact[215, 216, 352, 353]. The rate of formation of lymphocytes *in vivo* and *in vitro* can be measured through the incorporation

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of radiophosphorus; again the DNA is not renewed[224,354]. The extensive measurements, by many authors, of the life span of human erythrocytes, leucocytes and platelets have been reviewed[355]. For information on the very important subject of the polynucleotide metabolism of virus-infected bacteria (*i.e.*, on the multiplication of bacteriophage), we must refer to review articles[356–364] and to some of the recent experimental papers[89,365–373]. We also refer to the literature for work on labeled 'transforming factors'[374,375]. Further isotopic studies have been carried out on the nucleotide metabolism in virus-infected plants[194,376,377] and animals[378–380]. For experiments on the inhibition of virus growth by antagonists, *cf.*[194,195,197,198,205,208].

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CHAPTER XVI

SPECIAL TOPICS IN INTERMEDIARY METABOLISM

Chapters XI–XV have dealt with several problems in intermediary metabolism directly connected with the assimilation of the chief constituents of food. In this concluding chapter we shall discuss isotopic investigations aimed at elucidating the synthesis of some special, physiologically active substances in the tissues of animals and plants, their localization in the tissues, and, finally, their removal from the tissues. Naturally, no sharp boundary between the subject matter of this chapter and that of the earlier chapters can be drawn. It is also obvious that only a few important examples can be selected for discussion here although very many investigations indeed have been undertaken. The first section is devoted to a particular aspect of protein metabolism (*cf.* Chapter XIV).

1. Immunological Problems

The antibodies, which are found among the serum globulins, constitute a group of agents characteristic of the metazoa. In the many isotopic investigations of these substances, either labeled antigens or labeled antibodies were used[1–4]. Immunological methods make possible the specific determination of individual proteins in mixtures. The use of radioactivity gives extreme sensitivity in detection; it also enables us to follow the kinetics in the stationary state. Radioimmunological work is important not only in itself, but it also adds to our knowledge of the complicated problem of protein synthesis.

Radiochemical procedures are valuable for serological assays *in vitro*[5]. In determinations of the titer of antigen solutions or antisera by a 'radioreagent method' (p. 109), the sample is reacted with labeled antibodies (or antigens), and the activity of the precipitate is measured; no determination of nitrogen is necessary[6,7]. The dissociation of antigen–antibody complexes can be determined *in vitro* by radiochemical methods[8]. The titer of an antiserum against sheep erythrocytes was determined by the decrease in the amount of labeled antibodies of the same type taken up from a standard solution by the erythrocytes in the presence of the antiserum; in this way, a single standard solution can be used to assay a large number of unlabeled serum samples[9]. The location of unlabeled antibodies inside the cell has been determined by autoradiography, after reaction of the antibodies with labeled antigens[10]. Labeled antigens have also been analysed by autoradiography with agar plates[11]. The determination of antibodies by isotope dilution methods and by transmigration electrophoresis have already been mentioned (p. 24 and 112, respectively).

Radioactive antigens can be produced by coupling proteins through diazo-groups to the haptenes, ^{35}S-sulfanilic acid or ^{14}C-anthranilic acid [12–17], or by treating the proteins with di-2-chloroethyl sulfone[18]. Proteins can also be labeled by iodination in alkaline solution; the iodine is taken up primarily by the tyrosine (see p. 35). All these procedures introduce an 'external' label by adding a foreign constituent to the protein molecule.

Accordingly, the 'radioantigen' produced by these procedures is no longer completely identical with the unlabeled protein. The inhomogeneity of the labeled protein introduces a further complication; if a random distribution is assumed, it may be calculated that, after taking up an average of 9 iodine atoms per molecule, only 12 % of the protein molecules actually contain 9 iodine atoms; 6.5 % of the molecules have 6 or 12 atoms, and 2 % have as many as 16 or as few as 2. Some of the labeled molecules will therefore deviate more than the average from the normal behavior of the unlabeled protein[19]. In fact, quite marked deviations have been observed on labeling with sulfones, or by diazotization, for example[2, 3, 5, 18, 20–22]. Changes can be induced even by the relatively mild iodination procedure; however, provided the iodination is not carried too far—for instance, if an average of 1 atom of iodine only per molecule of protein is introduced—the changes are negligible[2, 3, 6, 18, 19, 22–30]. In contrast to the highly-substituted proteins, these iodine-poor proteins remain in the circulation for many days, without being deposited in the organs[25, 30–32]. The various serum proteins take up iodine at different rates[19]. The yield in iodination is improved if iodine monochloride is employed[33].

'Internal' labeling, achieved by biosynthesis of radioactive substances, produces 'genuine' antigens. In this case the labeling takes place by metabolic processes, as described in section *IV, 3*. The first such substance to be produced was the nucleoprotein ^{32}P-tobacco mosaic virus[34]. ^{14}C-polysaccharide from *Pneumococcus* has also been produced biosynthetically[35]. The immunological use of biosynthetic proteins of animal origin will be discussed later.

The possibility must be kept in mind that the radioelement may be separated from the antigen in the body, so that the radioactivity no longer remains a measure of the quantity of antigen present. If experiments with iodinated protein are protracted sufficiently, the iodine may be found in various undesired forms, *e.g.*, as iodinated peptides or amino acids, and even as free iodide[2, 22, 29, 36]. In addition, a re-incorporation of degradation products of internally labeled antigens into other antigenic substances, for instance other serum proteins, may occur (see below). Effects of these kinds can be more or less ruled out if external and internal labeling lead to the same results, as would be the case, for example, if a doubly-labeled antigen in the blood exhibited a constant ratio of ^{131}I to ^{14}C[30, 36–38]. Reference to double labeling by ^{131}I and ^{133}I will be made below.

The distribution of antigens among the various organs, and the location of the antigens within the cells have been studied[3]. Early workers in the field found that relatively large amounts of radioelements were present in the liver, spleen, and lungs[16, 34, 39]. Autoradiography has shown that ^{14}C-tobacco mosaic virus is localized in the cytoplasm of bone marrow, spleen, and liver[40]. Haurowitz demonstrated that the radioactivity of microsomes and especially of mitochondria persists for several months, but it is questionable whether the radioactivity at the end of this period was still indicative of the presence of antigen[14, 15, 41–43]. One test consists in 'washing out' with identical, but non-radioactive antigen; the radioactivity should be affected only if the two substances are really identical chemically[44].

The amount of bovine serum albumin remaining in the circulation of a normal rabbit as a function of time is shown in Fig. 27 (Knox and Endicott[24]). The rapid decrease during the first day reflects the equilibration of the plasma protein with the extravascular compartment[3, 45]. Antigen molecules which were 'damaged' during preparation and labeling may be removed by a sort of 'phagocytosis'[46]. Quite an appreciable loss of labeled protein occurs within the first two hours[46]. The slow decrease in foreign protein, which follows the rapid initial decrease, and continues for several days, is ascribed to the gradual degradation of the protein by metabolic processes. The precipitous decline after a week is brought about by the appearance of antibodies; this phenomenon was also noted by other workers[47–51a].

During the period of rapid decline owing to combination with the antibody, the antigen is no longer reactive but it can be detected by its radioactivity[24]. 'It is abundantly clear that when lightly-labeled proteins are injected, an antibody response results which leads to the rapid destruction of the bulk if not of all the antigen, and that di-iodotyrosine-containing peptides appear in the urine; further it is found that the rate of excretion of split products parallels the disappearance of the

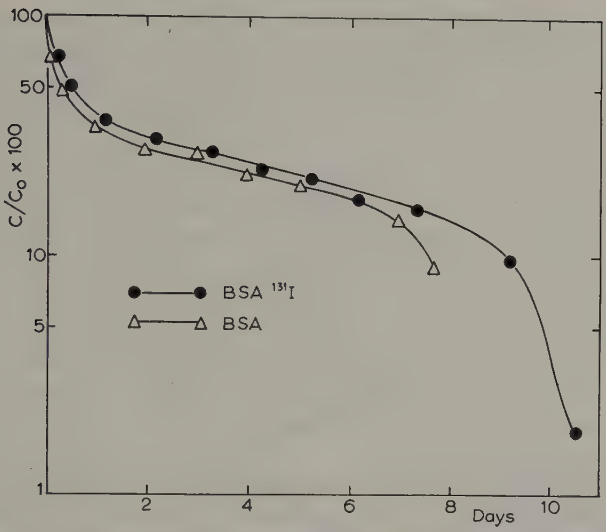


Fig. 27. The elimination of bovine serum albumin by an unimmunized rabbit. Lower curve: unlabeled albumin, determined serologically. Upper curve: radioiodine-labeled albumin, determined by radioactivity. (c_0 = original titer, c = titer remaining at any given time).

intact labeled material from the blood stream. These facts indicate that after union with antibody, or indeed as a result of such union, protein antigens are hydrolyzed by some proteolytic process'[3, 32].

This last phase is quite variable, however, and depends upon the animal species, the kind and amount of protein, and other circumstances. A labeled antigen is destroyed rapidly after injection into an immunized animal[32, 47, 48, 52–54]; the elimination of antigen labeled with radioiodine can be taken as an early indication of immunity[2]. The iodine of labeled bovine serum albumin was found in the liver, spleen, and lungs of an immunized rabbit soon after the injection[46].

The amino acid composition, molecular weight, electrophoretic mobility and antigenic activity of the antibodies indicate that they are globulins. Usually, but not invariably[55], they are γ-globulins[56]. After the 'microheterogeneity' of the γ-globulins had been demonstrated, it was found by partition chromatography on celite[57, 58] or by electrophoresis[57–59] that the serologically active globulins occurred in only a few of the fractions. Biosynthetically produced labeled antibodies can be purified by precipitation with antigen *in vitro*, followed by elution with unlabeled antibodies under appropriate conditions of temperature and acidity[60].

In pioneer experiments on the labeling of antibodies, it was found that rabbits and rats, which had been injected with antibodies against hemocyanin or *Pneumococcus* polysaccharides, did not incorporate ^{15}N-glycine into the antibodies. Labeled glycine *was* incorporated into the antibodies, however, if the antibodies were produced in the animal instead of being injected; in other words, incorporation takes place in actively, but not in passively immunized animals[61].

More precise studies can be carried out with radioactive compounds, namely tritium-[62], sulfur- and carbon-labeled antibodies. With mixtures of sulfur-labeled amino acids from yeast protein, it was confirmed that an antibody against bovine serum albumin, passively injected into rabbits, did not incorporate amino acids, and S-labeled antibodies produced in a donor animal retained the label when the antibodies were transferred to another animal. Accordingly, after administration of radioactive antibodies to an animal, which is not actively immunized, the total antibody concentration of the blood can be determined at any time by measuring the radioactivity[63–65].

Carbon-labeled lysine or phenylalanine was injected into rabbits which had been immunized against *Pneumococcus*. Thereafter, the antibodies and the 'normal' γ-globulin were isolated from the blood of the animals, and injected into other rabbits. The antibodies were stored, in part, in the organs (liver, kidney, spleen and bone marrow, for example), but their specific activity remained unchanged for three weeks (as long as it was followed) just as in the case of the experiments with radiosulfur. In other words, the antibodies and the radiocarbon disappear from the circulation of passively immunized animals at equal rates (12.5 % per day). We must again conclude that the antibodies do not exchange their amino acids, and that the label remains firmly attached to the molecules[66].

Since the quantity of normal globulin in the circulating fluid must remain nearly constant, the decrease in the amount of injected normal globulin is, in contrast to the behavior of the antibodies, reflected in a decrease in the specific activity of the normal globulin. The injected globulin disappears almost as rapidly as the antibody; the slight difference may be due to the fact that the amino acids released during degradation can be re-incorporated into normal globulin, but not into the antibody. Another portion of the amino acids is incorporated into other, serologically inactive, proteins[66] (Fig. 28). If an excess of antigen is administered to a passively immunized animal, the labeled antibody rapidly disappears from the circulation and the tissues, and is destroyed[51, 69].

(A comparison of antibody and normal serum globulin by radioactivity only is possible with double labeling. In one series of experiments, proteins containing ^{131}I and ^{133}I, respectively, were utilized[67]. Thus, complete equality of experimental conditions was ensured).

Actively-immunized acceptor rabbits have also been utilized. The

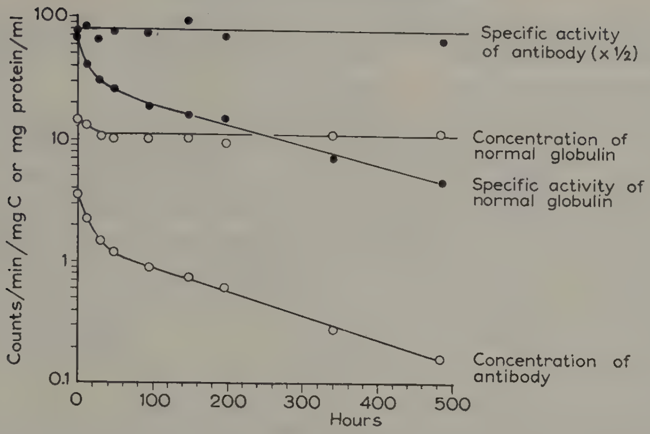


Fig. 28. Time course of the concentrations and specific activities of normal γ-globulin and antibody from rabbits, after injection into a normal rabbit[66]. (The injection consisted of 340 mg of antibody with 186 c.p.m./mgC, or of 495 mg of normal globulin with 200 c.p.m./mg C.).

specific activities of radioactive antibody or normal globulin decreased at the same rate (12.5 % per day); however, in this case the concentrations of both proteins remained constant. From the fact that the antibody radiocarbon disappeared at the same rate in both passively and actively immunized animals, it was concluded that this rate did not depend on the speed of *de novo* synthesis of the antibody[66].

The average life of the antibody varies from species to species (p. 231). It is 1–2 days in the mouse, 20 days in the cow, and hence has an intermediate value in the rabbit[63].

The absolute rate of formation of (unlabeled) antibody in the immunized animal must be proportional to the concentration of antibody, since the fractional decrease in specific activity per unit time is constant. (If the concentration of antibody changes—*i.e.*, if the animal is not exactly in a steady state—certain corrections must be applied.) The immunization of a 2.5 kg rabbit with only 2.5 mg of *Pneumococci* (only 10 % of which consisted of antigenic polysaccharide) led to the synthesis of 18 g of antibody in 19 days[66].

When rabbits immunized by subcutaneous rather than intravenous injection were provided with radioactive amino acids, radioactive antibodies appeared in the circulation only relatively slowly, and reached their maximal activity after approximately 50 hours[69]. The sites of antibody formation in the living rabbit were determined from the ^{14}C-antibody contents of the individual organs after treatment *in vivo* with labeled amino acids; allowance was made for extracellular antibody remaining within the separated organs after exsanguination by injecting ^{131}I-labeled antibody into the rabbit beforehand, so that at the time of death extracellular antibodies could be identified by their content of ^{131}I[70].

Radioactive γ-globulin can also be formed *in vitro*; tissue slices of spleen, lymph nodes, lung or bone marrow are active. If the tissues are derived from immunized animals, radioactive antibodies are also produced[58,69,71–75a]. In the case of rabbits actively immunized against two different proteins, incorporation of labeled amino acids into the two antibodies occurs simultaneously with hardly any mutual disturbances[76]. The production of γ-globulin from radioactive amino acids in tissue from chick mesenchyma and from HeLa tumor has been demonstrated[76a]. The investigation of the antibody synthesis in cells isolated from their original medium after *in vivo* sensitization has quite generally proved useful[75a].

Attempts have been made to elucidate the mechanism of the formation of antibodies from their precursors. From the experiments already described, as well as from further experiments[5,56,73,77], it may be concluded that the antibodies arise by *de novo* synthesis from amino acids. Other possibilities—such as the partial degradation of other serum proteins, with subsequent resynthesis, or a change in the configuration of the surface of other γ-globulins (altered molecular folding)—appear unlikely in view of the rapidity of the incorporation of free amino acids into the antibodies of immunized animals. As in the case of other serum proteins (p. 240), the isotope appears in the antibody of the rabbit within half an hour, and the maximum isotope content is attained after only a few hours[58,61]. If various fractions of labeled normal γ-globulins from a donor rabbit are injected into another rabbit which is in the process of being immunized, no radioactive antibodies appear in the latter animal; apparently the γ-globulin molecules, once formed, do not act as precursors of antibodies[58].

Green and Anker[56] have come to the conclusion that the incorporation of labeled amino acids into antibodies begins immediately upon administration of the antigen to non-immunized rabbits, and increases until precipitable antibodies appear in the circulation, *i.e.*, for several days. In one series of experiments they administered the labeled amino acid before the antigen; the specific activity of the antibody formed was then always less than that of the serum proteins. On the other hand, if the labeled amino acid was injected after the antigen, the radioactivity of the antibody proved to be particularly high.

Only small amounts of serologically-active antibodies could be found in the cells before they appeared in the circulation, despite the fact that the labeled amino acids were very quickly withdrawn from the blood to form the antibody. For this reason, it was postulated that a serologically-inactive precursor is formed first, and that the precursor is converted into the active antibody only near the end of an induction period, before it enters the circulation.

Taliaferro and Talmage, who carried out the induction and the antibody formation in different animals, came to a different conclusion[65, 78]. Three days after the injection of antigen into a donor rabbit, the spleen was homogenized and injected into a second rabbit; here serologically-demonstrable antibody was formed. The antibody became radioactive only when ^{35}S-amino acids were administered to the second animal; injection into the donor animal before removal of its spleen had almost no effect at all. The authors concluded that 'almost all of the sulfur-containing amino acids present in serum antibody are drawn from the amino acid pool during the rise of serum antibody and not during the induction period. The lack of incorporation of amino acids into antibody during the induction period may indicate that synthetic enzymes are developed during the induction period which, once present, begin to form antibodies. If only a short time elapses between the appearance of the suggested enzymes and the synthesis of antibodies, the rise of serum antibodies should give an accurate approximation of the rate of antibody formation[76].'

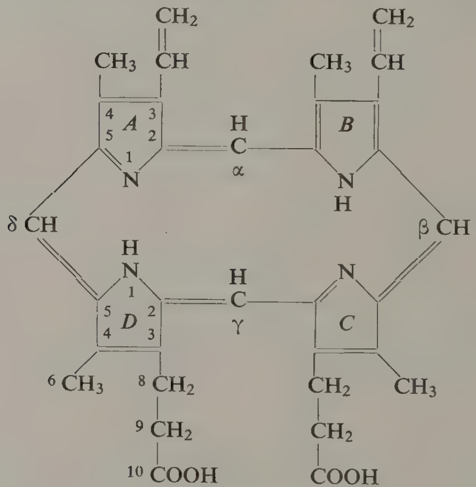
The template hypothesis was first applied to immunology by Breinl and Haurowitz. In its original form this hypothesis regarded the antibodies simply as negatives of the antigens; this would mean that antibody formation could no longer occur after the disappearance of the antigen[2, 79]. If the antigens are regarded as templates, no direct account is taken of the role of the nucleic acid in protein synthesis, but the concept can be extended in the light of recent knowledge (Chapter XIV). If the RNA serves as a template for the mass production of antibodies, the retention of (labeled) antigens in the body is not necessary for continued antibody formation[80,81]. The possibility, viewed favorably by some authors, that the antigen only affects the folding of the finished globulin chain, *i.e.* that the secondary structure of the protein only is shaped by the antigen, meets with difficulties as has been explained. Burnet stresses the parallelism between the induction of antibodies in higher animals and the induction of enzymes in bacteria[82]. Various features of the 'enzyme hypothesis' and of the 'template hypothesis' may well be reconciled[83, 75a].

Labeled atoms have been applied in various ways to elucidate the effects of irradiation on the formation of antibodies[75a, 84, 85]. The studies directed toward the use of antibodies as carriers of radioactivity for tumor diagnosis and therapy have been reviewed[86–88].

2. The Biosynthesis of Heme

The study of the synthesis of heme, the prosthetic group of hemoglobin and of several important enzymes, is in many respects a model of the application of isotopic methods to a biosynthetic problem. The study was initiated primarily by Shemin, Neuberger and their coworkers, and has already been pursued quite far[89]. The brief exposition which we shall present below follows, in the main, the extensive reviews of Shemin[90–93] and Popjak[94]. For shorter surveys see[95, 96]. For the chemistry of the porphyrins, we also refer to the reviews[97, 98].

It has been shown in the isotopic investigations that the complicated-looking molecule of protoporphyrin (heme minus iron) is formed from two, and only two, very simple substances of wide-spread occurrence in animal tissues, namely glycine and acetic acid. Both of these precursors are, of course, 'non-essential'; acetic acid can arise, for example, by the degradation of carbohydrates or fats along familiar pathways.



protoporphyrin.

The investigation was rendered much easier by the discovery of porphyrin synthesis *in vitro*, namely in duck blood. The blood of the birds contains erythrocytes which have not matured[99–101]. (See[102] for human blood). The use of blood instead of intact animals entails a tremendous saving of labeled material. Moreover enzyme systems are eliminated which may cause the various precursors under consideration to be mutually converted and thus obscure the results. For example, the whole animal—but not the blood—contains enzymes which form acetic acid from glycine. Yet the experiments *in vitro* probably give essentially identical results with those *in vivo*[103]. Rabbit bone marrow *in vitro* can also be used to study the biosynthesis of heme[104].

The point of departure was the very important observation that both man and the rat form protoporphyrin better—*i.e.*, in higher yield—from the (heavy) nitrogen of glycine than from that of any other simple compound [105–107]. The four pyrrole rings (*A*, *B*, *C*, *D*) are equally labeled; although the four rings are of two different types in respect to their side chains they all appear to be derived from the same precursor[108–112]. This precursor was later identified as porphobilinogen[107, 113, 114] (formula on p. 294).

After it had been determined that the nitrogen atom of glycine was specifically used for the synthesis of porphyrins, the fate of the carbon atoms during this biosynthesis was investigated with radiocarbon. It soon turned out that the carboxyl carbon was not incorporated, but the methylene carbon was; the quantitative comparison of the uptake of ^{15}N and ^{14}C revealed that twice as much methylene carbon as nitrogen was incorporated, *i.e.* the methylene carbon appears in eight positions of the molecule[108–112, 115–118].

A series of chemical degradation procedures was then developed, which made it possible to determine the activity of each individual carbon atom of the protoporphyrin (34 in all)[108–112, 118]. By these methods, the radioactivity of the heme formed from methylene-labeled glycine was localized in the four methene groups and in one position (C-2) of each pyrrole ring. The finding that half the methylene carbon atoms derived from the glycine were no longer bound to nitrogen might cause a suspicion that this carbon atom is utilized along two different pathways. This idea must be rejected, however. In the first place, no (labeled) precursors have been found in which the 2-positions of the pyrrole rings, but not the methene group, are labeled, or vice versa. In the second place, the specific activities of the carbon in both positions are always the same after introduction of radioglycine; this would be improbable, if the radiocarbon had had to pass through pools of different intermediates.

All of the other 26 carbon atoms are derived either from the methyl group or from the carboxyl group of acetic acid[109–112, 118, 119]. As in the case of the experiments with glycine, an equal distribution of the labeled atoms among the four pyrrole rings was found. Apart from the carboxyl groups, rings *C* and *D* contain just as much radiocarbon as do rings *A* and *B*. The carboxyl group remains unlabeled when $\overset{*}{C}H_3COOH$ is used, but becomes highly radioactive with $CH_3\overset{*}{C}OOH$. Each pyrrole ring exhibits the pattern of activity indicated in Fig. 29 (rings *C* and *D*). It is apparent that both 'sides' (right and left) of every ring arise from the same precursor. (Carbon atom 2, to the right of the nitrogen, is derived from glycine.)

The precursor might be either a C_3 or a C_4 compound, which would have to condense with glycine. A C_3 compound would have to be carboxylated at some stage to yield the propionate side chains of rings *C* and *D*; in the opposite case, a C_4 compound would have to be decarboxylated to produce all the methyl groups and the vinyl groups of the other two rings (*A* and *B*). The radioactivities of the carboxyl groups of protoporphyrin obtained from carboxyl-labeled acetic acid were identical with those of the neighboring methylene groups obtained from methyl-labeled acetic acid of the same specific activity. From the equality in the extent of dilution and from the relationships indicated in Fig. 29 it has been concluded that the acetic acid is always incorporated as a unit, and is utilized by way of a C_4 compound; consequently, the common precursory ring must contain contains one acetate and one propionate side chain in the β-positions. The methyl group of the porphyrin arises by decarboxylation of the former, and the vinyl group by decarboxylation and dehydrogenation of the latter.

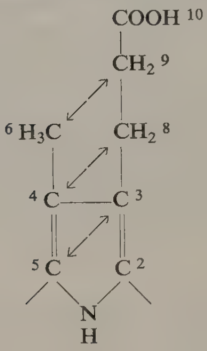


Fig. 29. Distribution of radioactivity in the substituted pyrrole rings *C* and *D* [109, 110]. The carbon atoms joined by arrows exhibit the same activities after administration of $\overset{*}{C}H_3COOH$, or of $CH_3\overset{*}{C}OOH$.

It was also presumed that the C_4 compound arises from acetate by way of the citric acid cycle. According to the scheme on p. 193, methyl-labeled acetate gives rise to α-ketoglutarate whose γ-carbon atom is just as active as the methyl carbon of the acetate, and whose other carbons are unlabeled. The 'middle' carbon atoms of the succinate and the other C_4 acids formed from the ketoglutarate are each half as active. If more labeled acetate is then taken up by condensation with the oxaloacetate later formed, and the cycle is repeated one or more times, the specific activity of the ketoglutarate is as shown in Table 12. The values given here are based on the assumption (which is not quite correct) that no dilution with inactive material takes place during the passage through the cycle. But whatever the dilution, the specific activities of the carbon atoms in equivalent positions of the α-ketoglutarate must remain equal.

The distribution of activity in the porphyrin (Fig. 29) is in agreement with Table 12 if it can be assumed that the C_4 precursor is asymmetrical, and that the carboxyl group of the porphyrin is derived mainly from the δ-carboxyl group of the α-ketoglutarate; the activity of this carboxyl group is small on application of $\overset{*}{C}H_3COOH$, and large on introduction of $CH_3\overset{*}{C}OOH$. If the C_4 precursor were symmetrical, positions 3 and 10 would be labeled equally. This is, however, not the case.

TABLE 12

SPECIFIC ACTIVITIES OF THE CARBON ATOMS OF α-KETOGLUTARIC ACID

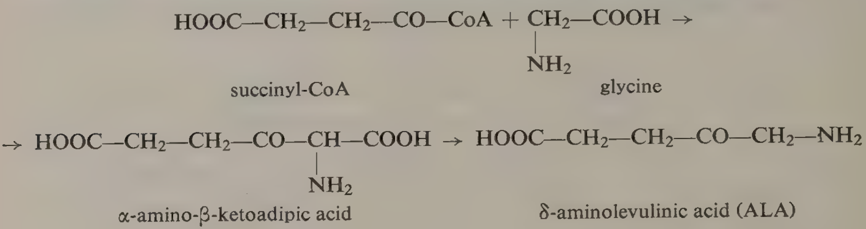
(percent of the specific activities of the acetate)

Group	*From* $\overset{*}{C}H_3COOH$				*From* $CH_3\overset{*}{C}OOH$			
	Number of turns of the cycle				*Number of turns of the cycle*			
	1	*2*	*3*	*∞*	*1*	*2*	*3*	*∞*
COOH	0	0	0	0	100	100	100	100
$CH_2 - \gamma$	100	100	100	100	0	0	0	0
$CH_2 - \beta$	0	50	75	100	0	0	0	0
CO $- \alpha$	0	50	75	100	0	0	0	0
COOH	0	0	25	50	0	50	50	50

The asymmetrical compound might be active succinate (succinyl coenzyme A). In this system, succinyl-CoA is formed primarily by oxidative decarboxylation of α-ketoglutaric acid, but the results of radiochemical analyses lead us to assume that a small portion must arise from some symmetrical compound—probably by esterification of succinic acid in the presence of an energy-rich nucleotide. The evidence for this is that position 3 exhibits a small but significant activity after introduction of $CH_3\overset{*}{C}OOH$[110, 120–123].

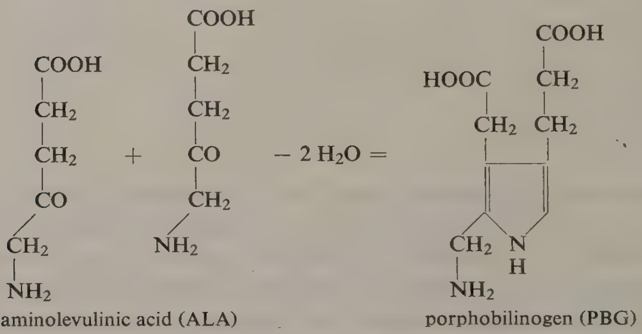
It is consistent with the proposed mechanism that malonate inhibits the incorporation into porphyrin of radiocarbon from methylene-labeled succinic acid, but not from carboxyl-labeled succinic acid. This is due to the fact that the carboxyl groups are lost in any case on passage through the citric acid cycle via malate and oxaloacetate; malonate inhibits these reactions. Radioactive succinyl-CoA, on the other hand, is produced by the reaction of carboxyl-labeled succinic acid with coenzyme A, a process not influenced by malonate. The radiocarbon of the methylene group, however, can be incorporated into succinyl-CoA either by direct reaction with coenzyme A, or after passage through the cycle, so that partial inhibition by malonate takes place[124]. The participation of citric acid and α-ketoglutaric acid in porphyrin synthesis has also been tested directly[125].

Succinyl-CoA probably first condenses with a pyridoxal phosphate derivative of glycine to form α-amino-β-ketoadipic acid[122, 123]. This β-keto acid is easily decarboxylated, with loss of the glycine carboxyl; the carbon of this group does not appear in the porphyrin. It is thought that a monopyrrole is formed from the decarboxylation product, δ-aminolevulinic acid (ALA)[113, 126]. Porphobilinogen (PBG) is considered to be the first monopyrrole; it occurs free in the urine of certain patients[127, 128].



$$\mathrm{HOOC{-}CH_2{-}CH_2{-}CO{-}CoA} + \underset{\mathrm{NH_2}}{\underset{|}{\mathrm{CH_2}}}\mathrm{{-}COOH} \rightarrow$$

$$\rightarrow \mathrm{HOOC{-}CH_2{-}CH_2{-}CO{-}}\underset{\mathrm{NH_2}}{\underset{|}{\mathrm{CH}}}\mathrm{{-}COOH} \rightarrow \mathrm{HOOC{-}CH_2{-}CH_2{-}CO{-}CH_2{-}NH_2}$$



The incorporation of radiocarbon from glycine or acetic acid into protoporphyrin is reduced in presence of a bank of inactive ALA. When labeled ALA was administered, on the other hand, the porphyrin was found to be labeled, and the radiocarbon was found in the proper positions; ALA-1,4-^{14}C gives the same type of labeling as succinate-1,4-^{14}C[100, 113, 126, 129–134]. The quantity of ALA utilized is not as large *in vivo* as *in vitro*; this difference may be a result of poor absorption[135, 136].

It is interesting to note that blood cells (intact, or hemolyzed with care) are required for the condensation of glycine with succinic acid, but that porphyrins are formed from ALA or PBG in homogenates as well[100, 124, 129–133, 137, 138]. It has even been possible to make soluble enzyme preparations from erythrocytes or from liver, which condense two molecules of (radioactive) ALA to one molecule of PBG[114, 132, 133, 139–141], and four molecules of PBG to one of protoporphyrin[114, 128, 141, 142]. In the absence of pantothenic acid, porphyrin synthesis from ALA can occur, but no ALA is formed from glycine and succinate[143]. The mechanism of

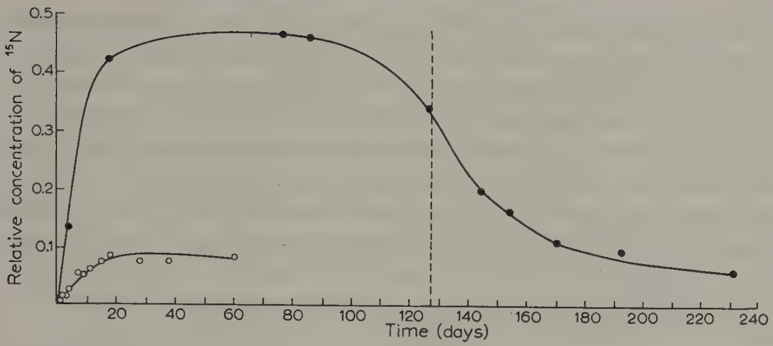


Fig. 30. The isotope content of human heme after administration of labeled glycine for 3 days. Upper curve: heme. Lower curve: protein of the red blood cells[106].

the synthesis of porphyrins from PBG[90, 91, 93, 94, 137, 144, 145] is not yet understood; an acceptable hypothesis would, of course, have to explain the loss of half of the nitrogen[145a]. Labeled ALA can also serve as a precursor of the porphyrin-like moiety of vitamin B_{12}[146–148].

The porphyrin of erythrocytes is not renewed; the heme, once formed, remains in the cells until they die, without taking part in any dynamic state[105, 106, 149]. Moreover, the 'used' porphyrin—in contrast to the iron—is excreted and not re-used. Accordingly, the isotope content of the circulating heme declines precipitously toward the end of the life-span of the labeled erythrocytes (Fig. 30). The precursors for the synthesis (glycine and acetic acid) are abundant in the organism; therefore a re-utilization of the porphyrin does not seem to be necessary.

The synthesis in the animal body of important substances other than porphyrins, and the complete oxidation of glycine carbon can also take place by way of ALA[113, 133, 150]. 'The condensation of either 'active' succinate or 'active' acetate provides a pathway whereby glycine can be oxidized to carbon dioxide and the intermediates produced in the cycle drawn off for the synthesis of other compounds. This is similar to the citric acid cycle, in which another two-carbon compound is oxidized to carbon dioxide and intermediates are produced which can be drawn off for synthesis. In the succinate-glycine cycle succinate is the catalyst instead of oxaloacetate'[151].

We shall refer only briefly to the studies on chlorophyll. The biosynthesis of this substance has not yet been elucidated completely[152–155]. Experiments on *Chlorella*[156] and on onion leaves[157–159] have shown that acetic acid and glycine serve as precursors in this case as well. The acetic acid can, of course, be derived from glucose. The carboxyl carbon of glycine is also reported to be incorporated into the chlorophyll rings[156].

The smallness of the effect may indicate that it is due to some secondary reaction[396]. Part of the carbon of glycine or acetate is located in the ester methyl of the methyl pheophorbide[156]. This methyl carbon atom can also be derived from labeled formate[160].

It has also been suggested—contrary to earlier beliefs—that plants (*Chlorella*) do not make chlorophyll b from chlorophyll a. Rather, both forms are derived from a common precursor; the methyl pheophorbide obtained from either pigment had the same specific activity after the introduction of labeled acetic acid or glycine[156]. More complicated relationships were observed in certain experiments with higher plants[157, 159, 161, 162].

After the synthesis of chlorophyll has been completed, plants can still incorporate radiocarbon into the chlorophyll. Thus chlorophyll, like most constituents of living tissue, but unlike heme, is in a dynamic state [163]. A half-life of 13 days was found for the chlorophyll carbon of mature tobacco plants[159]; the nitrogen within chlorophyll of grasses exchanged to the extent of 95 % within three days[164, 165]. The carbon of carotene (p. 299) is also renewed rapidly[157, 159, 161, 162, 166]. The different constituents of chlorophyll do not appear to be renewed independently[157, 159, 161, 162]. No exchange, *in vivo*, was observed between radiomagnesium and chlorophyll[167].

After administration of labeled glucose to onion leaves, the phytol also proved to be radioactive. The specific activity was close to that of the chlorophyll[137, 138, 141, 142].

3. The Biosynthesis of Cholesterol

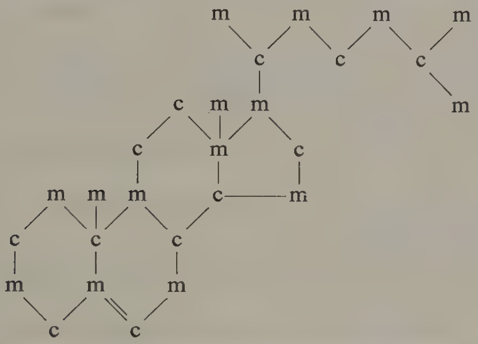
The biosynthesis of cholesterol has not yet been elucidated as fully as that of heme, but great progress has been made in the past few years. The following survey is largely based upon the reviews by Bloch and Popjak [94, 168–176].

Mammals are capable of synthesizing cholesterol as well as heme. Exogenous cholesterol is, however, also utilized. It was found quite early that the deuterium content of the cholesterol of an animal quickly approaches that of the blood; this made it seem probable that the cholesterol is built up from small molecules[177]. The half-life of deuterium in serum cholesterol is approximately one week[178, 179] (*cf.* p. 142). No large amount of carbon from (labeled) cholesterol appears in the respiratory CO_2[180].

Investigations with labeled carbon revealed that cholesterol, in contrast to heme, can be synthesized from a single precursor, acetate; both carbon atoms of the acetate are used[119, 181–185]. By using doubly-labeled acetate, $^{13}CH_3{}^{14}COOH$, it was found that the methyl and carboxyl carbons enter the 27-positions of the cholesterol molecule in the ratio 15 : 12 [186].

Most investigations of cholesterol synthesis are carried out on liver slices which are incubated aerobically with labeled acetate[184], but experiments with homogenates and cell-free extracts are also useful[187–189]. As in the case of heme, it was necessary to work out degradative methods which made it possible to measure the activity of each carbon atom separately[94, 190]. The first step consists in removing the side chain from the ring system.

When the degradative methods were applied to cholesterol produced from methyl-labeled or from carboxyl-labeled acetate, it was found that some of the carbon atoms of cholesterol acquired a label from the methyl carbon, and others from the carboxyl carbon, but none from both carbon atoms. Denoting the methyl carbon by m and the carboxyl carbon by c, we may write[190]:



Origin of the carbon atoms of cholesterol.

The cholesterol skeleton may be looked upon as being made up of terpene units, and hence, of isoprene (isopentane) groups[191]; thus the biosynthetic pathways are considered to pass through an isopentane derivative. One fundamental question is, then, how do the isoprene groups arise from acetate? An hypothesis about the biogenesis of rubber served as a point of departure.

The direct polymerization of isoprene to rubber cannot be assumed, since isoprene has never been found in plants, and is, indeed, not utilized by them[192]. Bonner and his colleagues have shown that the yield of rubber from *Hevea* is increased by the administration of glycerol, acetate, acetone, acetoacetate, or β, β-dimethylacrylate (= β-methylcrotonate) [193–195]. If a large quantity of labeled acetate is administered to guayule (*Parthenium argentatum*), the specific activity of the rubber approaches that of the acetate, *i.e.*, the rubber is derived almost entirely from the acetate[196]. Radioactive acetate is incorporated into rubber even *in vitro*, in latex[197,198]. Other radiocarbon studies have been carried out with the rubber plant kok-sagys[199]. Carotene, which likewise consists of isoprene units, is also synthesized from labeled acetate[199–206] (see below). Terpenes have been obtained from labeled dimethylacrylic acid[207].

After radiophotosynthesis with *Hevea*, labeled latex appears first in the leaves; it was concluded that the precursors are also formed in the leaves. When the trees were returned to a normal atmosphere after radiophotosynthesis, the rubber lost its activity rapidly, without decreasing in total quantity; apparently the rubber takes part in metabolism[208]. A dynamic state of the terpenes of *Pinus sylvestris* has also been reported[209].

What, then, is the nature of the isopentane derivative which can be reduced, dehydrated, decarboxylated, and polymerized to form rubber? Many plant tissues (as well as animal tissues; *cf.* p. 214) condense acetyl-coenzyme A enzymatically to acetoacetate; this is most probably the first step[210]. The subsequent reactions are still not definitely known.

One possibility, for example, is the scheme of Bonner, in which condensation of the acetoacetate with another molecule of acetate to form β-hydroxy-β-methylglutaric acid (HMG),

$$CH_3COCH_2COOH + CH_3COOH \rightarrow HOOCCH_2C(OH)(CH_3)CH_2COOH$$

followed by dehydration, gives rise to methylglutaconic acid, which loses CO_2 to form methylcrotonic acid

$$HOOCCH_2C(CH_3) : CHCOOH \rightarrow (CH_3)_2C : CHCOOH$$

An enzyme system has actually been obtained from young flax plants, which in the presence of ATP and coenzyme-A converts acetate to methylcrotonate by way of acetoacetate and HMG[211].

It has been assumed that the first few steps in the synthesis of sterols are basically the same as those in rubber synthesis[172, 212]. Liver and liver extracts do in fact form HMG and methylcrotonate from acetyl-coenzyme A[213–216]. Radiocarbon from HMG and methylcrotonate is incorporated into cholesterol (and into squalene; see below) *in vivo* and—although there is some controversy about this—possibly *in vitro* as well[207, 213, 217–221].

The assumption that acetoacetate is an intermediate is supported by the fact that acetoacetate radiocarbon is incorporated into cholesterol by rat liver even in the presence of a large excess of unlabeled acetate; dilution of the isotope would have occurred if the acetoacetate had first been broken down to acetate[222, 223].

Not only the acetate, but also the acetoacetate is utilized (by mammalian liver and also by yeast) for HMG synthesis in the form of the thioester with CoA[224]. Using labelled acetyl-coenzyme A, it was demonstrated that the coenzyme released during the condensation originally had been attached to acetate rather than to acetoacetate[225,226].

The enzyme systems of liver and heart seem to effect reactions of HMG which are somewhat different from those first postulated by Bonner, however. The HMG first loses CO_2, and dehydration of the product, β-hydroxyisovaleric acid (HIV), to β-methylcrotonic acid follows[224,227,228].

$$\mathrm{HOOCCH_2C(OH)(CH_3)CH_2CO{—}CoA} \rightarrow \mathrm{(CH_3)_2C(OH)CH_2CO{—}CoA} \rightarrow$$
$$\rightarrow \mathrm{(CH_3)_2C:CCO{—}CoA}$$

No matter which mechanism is the right one, the methyl- or carboxyl-carbons of the acetate (m and c) will be distributed in the isopentane skeleton according to the scheme m_2 : c—m—c; this distribution corresponds to that of the side chain of cholesterol. There is nevertheless considerable doubt whether methylcrotonic acid lies on the main pathway of sterol synthesis[229].

It has recently been discovered that liver homogenates very easily incorporate 'mevalonic acid' (β, δ-dihydroxy-β-methylvaleric acid) and its lactone into cholesterol and squalene; the carboxyl carbon of the C_6 compound is lost in the process[221,230–233]. On the other hand, an inactive bank of mevalonic acid inhibits the incorporation of acetate radiocarbon into cholesterol by liver homogenates and liver slices[230,234].

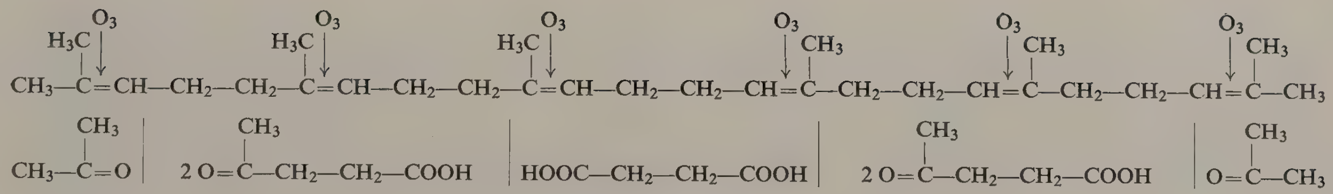
It appears now on the basis of experiments with radiocarbon that mevalonic acid is produced by reduction of the thioester of coenzyme A with HMG[229,229a,258a]. TPNH serves as reducing agent in the presence of the specific enzyme HMG reductase.

$$\mathrm{HOOCCH_2C(OH)(CH_3)CH_2CO{—}CoA} + \mathrm{2TPNH} + \mathrm{2H^+} \rightarrow$$
$$\rightarrow \mathrm{HOOCCH_2C(OH)(CH_3)CH_2CH_2OH} + \mathrm{CoA} + \mathrm{2TPN}$$

Mevalonic acid was found to be a precursor of β-carotene in *Phycomyces blakesleeanus*[236,237] and of α-pinene in pine[238], but not of β-carotene in maize seedlings[239] (see also p. 298). In regard to its incorporation into rubber in *Hevea* latex *in vitro*, the situation is still obscure[240–242a].

In the attempt to conceive the whole cholesterol molecule—and not just the side chain—as being composed of isoprene residues, it is assumed, in agreement with an old hypothesis of Robinson and Heilbron, that the open-chain triterpene, squalene ($C_{30}H_{50}$), occurs as an intermediate. Various pathways for the cyclization of squalene have been suggested by Woodward and Bloch[171,243,244], Ruzicka[191] and Tschesche[173] (see[397]).

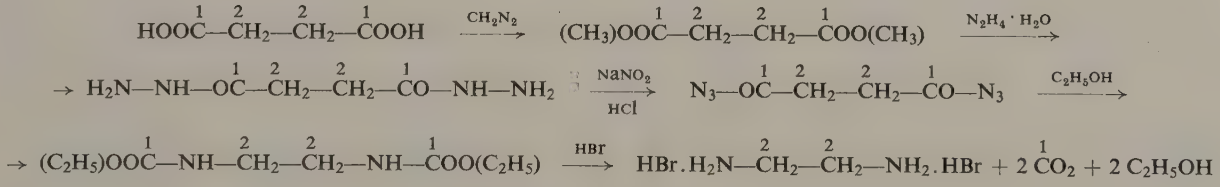
Radioactive squalene has in fact been isolated from rat livers after feeding radioactive acetate[245]. In liver slices and extracts as well, squalene is produced from acetate or from mevalonic acid[220,221,246–248] (p. 298).



$$CH_3\text{—}\overset{H_3C}{C}{=}CH\text{—}CH_2\text{—}CH_2\text{—}\overset{H_3C}{C}{=}CH\text{—}CH_2\text{—}CH_2\text{—}\overset{H_3C}{C}{=}CH\text{—}CH_2\text{—}CH_2\text{—}CH{=}\overset{CH_3}{C}\text{—}CH_2\text{—}CH_2\text{—}CH{=}\overset{CH_3}{C}\text{—}CH_2\text{—}CH_2\text{—}CH{=}\overset{CH_3}{C}\text{—}CH_3$$

$$CH_3\text{—}\overset{CH_3}{C}{=}O \;\Big|\; 2\,O{=}\overset{CH_3}{C}\text{—}CH_2\text{—}CH_2\text{—}COOH \;\Big|\; HOOC\text{—}CH_2\text{—}CH_2\text{—}COOH \;\Big|\; 2\,O{=}\overset{CH_3}{C}\text{—}CH_2\text{—}CH_2\text{—}COOH \;\Big|\; O{=}\overset{CH_3}{C}\text{—}CH_3$$

The degradation of squalene.

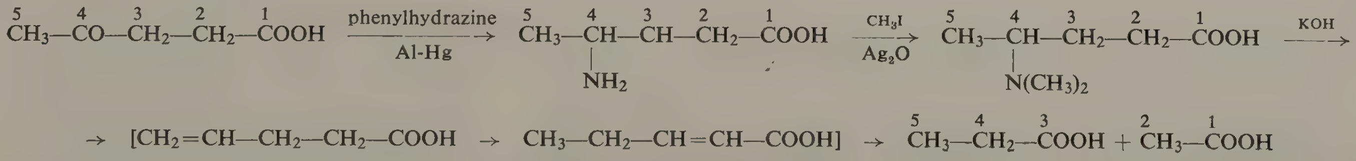


$$HOO\overset{1}{C}\text{—}\overset{2}{C}H_2\text{—}\overset{2}{C}H_2\text{—}\overset{1}{C}OOH \xrightarrow{CH_2N_2} (CH_3)OO\overset{1}{C}\text{—}\overset{2}{C}H_2\text{—}\overset{2}{C}H_2\text{—}\overset{1}{C}OO(CH_3) \xrightarrow{N_2H_4 \cdot H_2O}$$

$$\rightarrow H_2N\text{—}NH\text{—}O\overset{1}{C}\text{—}\overset{2}{C}H_2\text{—}\overset{2}{C}H_2\text{—}\overset{1}{C}O\text{—}NH\text{—}NH_2 \xrightarrow[HCl]{NaNO_2} N_3\text{—}O\overset{1}{C}\text{—}\overset{2}{C}H_2\text{—}\overset{2}{C}H_2\text{—}\overset{1}{C}O\text{—}N_3 \xrightarrow{C_2H_5OH}$$

$$\rightarrow (C_2H_5)OO\overset{1}{C}\text{—}NH\text{—}\overset{2}{C}H_2\text{—}\overset{2}{C}H_2\text{—}NH\text{—}\overset{1}{C}OO(C_2H_5) \xrightarrow{HBr} HBr.H_2N\text{—}\overset{2}{C}H_2\text{—}\overset{2}{C}H_2\text{—}NH_2.HBr + 2\,\overset{1}{C}O_2 + 2\,C_2H_5OH$$

The degradation of succinic acid.



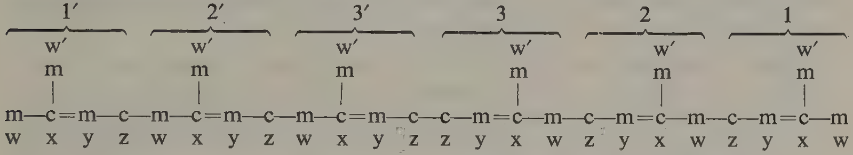
$$\overset{5}{C}H_3\text{—}\overset{4}{C}O\text{—}\overset{3}{C}H_2\text{—}\overset{2}{C}H_2\text{—}\overset{1}{C}OOH \xrightarrow[Al\text{-}Hg]{phenylhydrazine} \overset{5}{C}H_3\text{—}\underset{NH_2}{\overset{4}{C}H}\text{—}\overset{3}{C}H\text{—}\overset{2}{C}H_2\text{—}\overset{1}{C}OOH \xrightarrow[Ag_2O]{CH_3I} \overset{5}{C}H_3\text{—}\underset{N(CH_3)_2}{\overset{4}{C}H}\text{—}\overset{3}{C}H_2\text{—}\overset{2}{C}H_2\text{—}\overset{1}{C}OOH \xrightarrow{KOH}$$

$$\rightarrow [CH_2{=}CH\text{—}CH_2\text{—}CH_2\text{—}COOH \rightarrow CH_3\text{—}CH_2\text{—}CH{=}CH\text{—}COOH] \rightarrow \overset{5}{C}H_3\text{—}\overset{4}{C}H_2\text{—}\overset{3}{C}OOH + \overset{2}{C}H_3\text{—}\overset{1}{C}OOH$$

The degradation of levulinic acid.

Squalene is also a precursor of cholesterol[220, 245, 248]. Certain kinds of sharks, in whose livers squalene abounds, are useful for the biosynthetic preparation of labeled squalene with the 'correct'steric configuration[249]; abiosynthesis produces a mixture of isomers, some of which are biologically inactive.

In order to test the hypothesis further, the distribution of radiocarbon in squalene formed from labeled acetate was determined by Cornforth and Popjak[250] (see opposite page).



$$\begin{array}{cccccccccccccccccccccccc}
\multicolumn{4}{c}{\overbrace{\hspace{6em}}^{1'}} & \multicolumn{4}{c}{\overbrace{\hspace{6em}}^{2'}} & \multicolumn{4}{c}{\overbrace{\hspace{6em}}^{3'}} & \multicolumn{4}{c}{\overbrace{\hspace{6em}}^{3}} & \multicolumn{4}{c}{\overbrace{\hspace{6em}}^{2}} & \multicolumn{4}{c}{\overbrace{\hspace{6em}}^{1}} \\
 & w' & & & & w' & & & & w' & & & & & w' & & & & w' & & & & w' & \\
 & m & & & & m & & & & m & & & & & m & & & & m & & & & m & \\
 & | & & & & | & & & & | & & & & & | & & & & | & & & & | & \\
m- & c= & m- & c- & m- & c= & m- & c- & m- & c= & m- & c- & c- & m= & c- & m- & c- & m= & c- & m- & c- & m= & c- & m \\
w & x & y & z & w & x & y & z & w & x & y & z & z & y & x & w & z & y & x & w & z & y & x & w
\end{array}$$

Schematic formula of squalene (following the notation of Klyne).

The squalene was first degraded with ozone to acetone, levulinic acid and succinic acid. The acetone was degraded by the iodoform reaction, and the succinic acid, by the Curtius rearrangement.

It was found that the carboxyl carbons of the succinic acid are derived from the carbon atoms y 3 and y 3′, and the methylene carbons from z 3 and z 3′.

Every molecule of levulinic acid derives each carbon atom from the analogous position of the isoprenoid units, *i.e.*, from carbon atoms y, z, w, x and w′, carbon atoms x 2, x 2′, x 3 and x 3′ yielding the carbonyl carbon, and y 1, y 1′, y 2, and y 2′ yielding the carboxyl carbon. The levulinic acid was analyzed by reducing the phenylhydrazone with aluminum amalgam to 4-aminopentanoic acid. Methylation of this amino acid yielded the betaine. When fused with potassium hydroxide (350°) this gave acetic acid from the carboxyl and α-carbon atoms, and propionic acid from the β-, γ- and δ-carbons. The propionic and acetic acids were separated by chromatography, and degraded stepwise.

Degradation of the levulinic acid alone should yield unequivocal information about the distribution of acetate carbon in the squalene, since there are only 5 'types' of isoprene carbons in squalene, and all 5 types are contained in the levulinic acid. In experiments with methyl-labeled acetate it was shown that the carbon atoms w, w′ and y of the isoprene units had the same activity. Atoms x and z must arise from the acetate carboxyl group. Their (slight) activity in these experiments with methyl-labeled acetate was ascribed to the fact that $CH_3\overset{*}{C}OOH$ yields a small amount of $CH_3\overset{*}{C}OOH$ by way of the citric acid cycle[251].

In experiments with mevalonic acid-2-^{14}C-5-di^3H added to yeast extracts, some insight into the mechanism of squalene biosynthesis from the

5-carbon precursor has been obtained. The ratio of the isotopes in the squalene produced was the same as that in the mevalonic acid; the conclusion was that the condensation cannot be accompanied by oxidation at the δ-carbon atom[252]. In further experiments with rat liver homogenates and mevalonic acid-2-^{14}C it was found that all carbon atoms maintain their individuality during biosynthesis and that carbon atom 2 enters the w-position of the squalene[231,233,253–255]. Phospho-mevalonic acid has been implicated as an energy-rich intermediate in the production of squalene from mevalonic acid, and suggestions about the mechanism of the further steps have been made[256–258a].

In all these experiments only the existence of a pathway: acetate → acetoacetate → squalene → cholesterol is proved. But this need not be the only or the principal pathway. For example, squalene might not be the immediate precursor of cholesterol, but only be in equilibrium with the precursor[216]. Nevertheless, we may presume that at least the main features of the pathway of cholesterol synthesis have been elucidated by these studies[259]. From the mentioned experiments on β-carotene it appears that the distribution of methyl and carboxyl carbons of acetate in the chain is similar to that in squalene (p. 299). This is not true, however, of the tomato pigment, lycopene[260].

Cholesterol may be formed from squalene by way of lanosterol and zymosterol[191]. By the use of radiocarbon the formation of lanosterol from squalene[261], and of cholesterol from lanosterol[262,263] and from zymosterol[264,265], have been demonstrated in the rat. Within 10 minutes after injection of labeled acetate into the rat, the liver of the animal was found to contain lanosterol of high specific activity; is is apparent that this sterol is not only quickly formed, but also quickly consumed, so that its steady-state concentration is rather small[266]. Such experiments, however, do not prove that this is the only physiological pathway for cholesterol synthesis.

The optimal composition of the medium for cholesterol synthesis by liver homogenates has also been investigated with labeled atoms[267]. The physiological aspects of cholesterol metabolism have been frequently studied[174,268]. For instance, the decrease in the biosynthesis of cholesterol on addition of cholesterol to the diet has been followed[269–276]. The rates of entry of radiocarbon from acetate into the cholesterol of various organs were compared; the cholesterol of the brain is the last to take up the labeled carbon[277–279]. The use of hydrogen-labeled substances has been advocated for the investigation of the absorption and distribution of sterols[280–282].

The biosynthesis of ergosterol (in yeast) has not yet been investigated as thoroughly as that of cholesterol, but it has been found that the ring system is built up in a very similar manner[171, 283–287]. Carbon atom 28 in the side chain is not derived from acetate, but rather from labile methyl[288–290].

The synthesis of other sterols and the mutual interconversions of sterols (*e.g.*, sex hormones) can be studied with labeled atoms. The remarkable possibilities of investigations with isotopes have so far been exploited only to a relatively slight extent [168–170, 173, 174, 291–300].

As far as the localization of labeled sterols in the organs of animals is concerned, one reviewer has come to the conclusion: 'None of the labeled steroids seems to concentrate to any extent in the organ on which it has its physiological effect. The possibility of using tissue selectivity of a radioactive steroid as a basis for localized radiotherapy seems very remote at the present stage of our knowledge'[297].

4. The Biosynthesis of Lignin*

According to the concepts of Freudenberg, lignin is a dehydrogenated, polymerized coniferyl alcohol. Coniferin, the D-glucoside of coniferyl alcohol, has been shown to be present in large quantities in cambial sap. One or two mg of synthetic coniferin-1-^{14}C were introduced beneath the bark of a two-year old spruce sapling, and the wound sealed up. After the coniferin had been left for several months, it was shown by autoradiography of longitudinal and transverse sections that the radioactive material had been deposited in the shape of a thin cylinder in the springwood, at heights up to 3 cm above the site of implantation. The radioactive material produced was insoluble in water, and could no longer be separated from the lignin. A hydrochloric acid lignin prepared from the wood lignin contained all the radioactivity. Formaldehyde made from the radioactive lignin proved to be radioactive, though not as active as was expected[301–305].

According to Freudenberg, the first step in the synthesis of lignin must be the enzymatic cleavage of coniferin by β-D-glucosidases. Experiments showed that 90 % of L-coniferin-1-^{14}C remained soluble after implantation, and that the substance was not attacked by β-D-glucosidase[306].

Thus D-coniferin (or products of its transformation) was indeed taken up and incorporated, but it was not clear whether a genuine synthesis of lignin had taken place. Freudenberg therefore carried out model experiments with the aglucone of coniferin, coniferyl alcohol. A dehydrogenase from mushrooms produced *in vitro* from coniferyl alcohol a so-called

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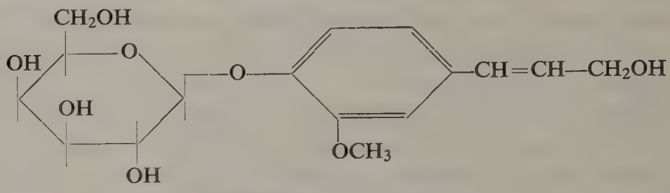
* This section has been contributed by Dr. G. Billek.



coniferin.

DHP (dehydrogenation polymer), which resembled an alcohol-soluble lignin in many respects[306]. By stopping the reaction before completion, it was possible to isolate the initial polymerization products as well-defined dimers, the 'secondary building stones' of lignin (dehydro-di-coniferyl alcohol and -aldehyde, DL-pinoresinol, β-coniferyl ether of guiacylglycerol, and some coniferyl aldehyde); these products were later discovered in cambial sap[307].

An active DHP prepared from coniferyl alcohol-1-^{14}C yielded inactive isohemipinic acid upon methylation and oxidative degradation; apparently a coumaran ring, rather than a chroman ring, had been formed[308].

To prove that a substance made available to the plant has been converted to lignin, various degradation products of the radioactive material formed must be investigated. High yields of vanillin are obtained by alkaline oxidation of the lignin of *Gymnosperms* and of the lignin of *Angiosperms*. The latter moreover produces syringaldehyde. The lignin of monocotyledonous plants yields additionally *p*-hydroxybenzaldehyde. It was possible by this method to confirm the conversion of many compounds into polymers which then yielded the above-mentioned aldehydes[309, 310]. Phenylalanine, benzoic acid, anisic acid, *p*-hydroxybenzoic acid, vanillic acid, protocatechuic acid, ferulic acid* and cinnamic acid, for example, were all made available to the plant, and the radiocarbon introduced was subsequently, to a greater or smaller extent, recovered in the aldehydes. However, compounds with no aromatic nucleus, such as shikimic acid, have also proved to be possible precursors of lignin[312].

We may presume with considerable assurance that aromatic compounds in plants are derived from carbohydrates, and that shikimic acid is an important intermediate in their synthesis. Eberhardt and Schubert[313] administered shikimic acid-2,6-^{14}C to sugar cane and obtained vanillin-

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* Ferulic acid-2-^{14}C[311] was allowed to react with the leaves and young shoots of *Helianthus annus*, *Triticum vulgare* and *Zea mays*. The presence of radioactive caffeic acid, chlorogenic acid and scopoline (7-glucosido-6-methoxy-7-hydroxycoumarin) could then be demonstrated in the methanol soluble fraction. This indicates that derivatives of cinnamic acid occupy a central place in the biogenesis of many plant substances.

2,6-^{14}C by oxidative degradation of the lignin. This indicates that the shikimic acid is incorporated without rearrangement of the carbon atoms of the ring. On the other hand, experiments on the formation of the methyl ester of *p*-methoxycinnamic acid by the fungus *Lentinus lepideus* made it appear probable that shikimic acid is converted to cinnamic acid derivatives by way of the *p*-hydroxyphenylpyruvic acid in this organism[314]; it was natural, then, to consider *p*-hydroxyphenylpyruvic acid as a possible precursor of lignin. Nord has demonstrated that *p*-hydroxyphenylpyruvic acid with ^{14}C in the carboxyl group is taken up by sugar cane (*Saccharum officinarum*) and transformed in such a way that a large fraction of the activity appears in the lignin[315, 316].

Similar experiments have been performed by Kratzl and Billek[317, 317a], employing spruce (*Picea excelsa*) instead of sugar cane. After feeding 4-hydroxyphenylpyruvic acid-3-^{14}C[317b], the striking result was obtained that the activity of typical lignin degradation products (*e.g.* vanillin and vanilloyl methyl ketone) was very low even though a highly active lignin could be isolated. Thus an artificial copolymer instead of real lignin had been formed in spruce. The difference between these results and the findings of Nord[315, 316] could be explained by Neish[318] who showed that only grasses, *e.g.* wheat (*Triticum vulgare*) or sugar cane as used by Nord[315, 316], convert 4-hydroxyphenylpyruvic acid into lignin whereas spruce[317], buckwheat (*Fagopyrum tartaricum*) and salvia (*Salvia splendens*) are unable to do so.

In order to avoid the loss of two side chain carbons, Kratzl employed ethanolysis for the degradation of lignin. When the lignin is treated with an alcoholic solution of HCl, the well-defined phenylpropane derivatives (so-called 'Hibbert building stones') α-ethoxypropioguiacone, vanilloyl methyl ketone, guiacylacetone, α-hydroxypropioguiacone, and small amounts of vanillin are obtained. A paper chromatographic method has been worked out for separating these substances[319, 320]. To exclude possible rearrangements occurring during the biogenesis or the degradation, two coniferins with different labels were synthesized: coniferin-2-^{14}C[321] and coniferin-3-^{14}C[322]. These substances were administered to plant tissue in the usual way, and all of the Hibbert building stones, in labeled form, were detected by autoradiography[307, 323, 324].

For exact measurements of activity and, hence, for constructing a balance sheet of the activity, this degradation has also been performed on a larger scale. It was proved by isolation of labeled vanilloyl methyl ketone from a coniferin-3-^{14}C implant (in spruce) that the side chain of coniferin had been so altered in the lignin side chain that it became susceptible to ethanolysis[325].

However, since this procedure would not reveal possible rearrangements during the incorporation and the degradation, the Hibbert substances were labeled in definite positions[326], and the stepwise degradation of the side chain was followed in order to locate the active positions[327, 328].

When such a degradation was performed on the isolated Hibbert substances, information could be obtained about the synthesis of the side chain, provided that the plant tissues were treated not only with C_6—C_3 compounds, as before, but also with C_6—C_1 compounds. Several such compounds (methyl-^{14}C-cresol, carbinol-^{14}C-vanilloyl alcohol, carbonyl-^{14}C vanillin, carboxyl-^{14}C vanillic acid, and their glycosides) have already been synthesized[326]. The structure of the side chain can also be investigated by sulfonating the lignin and degrading the lignosulfonic acid with alkali to yield vanillin and acetaldehyde. When coniferin-2-^{14}C had been implanted, the acetaldehyde was active, and the vanillin almost inactive; with coniferin-3-^{14}C, the distribution of activity was reversed. These results suggest that extensive rearrangements do not take place in the side chain[329].

These experiments on intact plants were supplemented by experiments carried out *in vitro* on more or less altered plant tissue. Of all the plant tissues which had been treated in various ways, only that which had been (briefly) heated completely lost the capacity to fix labeled coniferin. There is no doubt, therefore, that an enzyme system is involved in the incorporation of coniferin; this is in agreement with the results of Freudenberg[327].

Although the experiments referred to deal primarily with spruce lignin, which has been investigated most extensively, labeled substances have also been prepared which serve as precursors of the lignin of dicotyledons; these include syringin-2-^{14}C [330] and syringin-3-^{14}C [321]. *p*-Coumarol glucosides labeled in the 2- or 3-position have also been synthesized; they may be precursors of the lignin of monocotyledons[321, 330].

The incorporation into lignin of radiocarbon from CO_2 has also been followed. Active vanillin was obtained by subsequent oxidative degradation of the lignin isolated from the plant. The time course of the process and the extent of incorporation of 'new' carbon into the growing lignin were studied[331, 332].

The biosynthesis of the methoxyl groups of lignin has been investigated in tobacco (*Nicotiana rustica*) with serine-3-^{14}C, ^{14}C-formaldehyde, glycine-1-^{14}C and glycine-2-^{14}C[333].

Recently Freudenberg[334] was able to show that phenylalanine is converted into coniferin in tissues of spruce. This fact proves the important role of coniferin as the key intermediate in the biosynthesis of lignin.

5. The Degradation of Foreign, Physiologically-Active Substances by the Animal Body (Detoxication)

The investigation of the degradation of foreign organic substances—the so-called 'fate' of these substances in the organisms—often makes it easier to understand their mode of action. The study of the fate of a physiologically-active material usually goes hand in hand with the investigation of the localization of the substance and of its degradation products. In work of this type, the material is administered in labeled form, and the nature of the stored or excreted labeled substance is examined. The metabolism generally depends to a large extent on the dose (*cf.* Chapter X). In the following discussion, we shall deal primarily with the fate of physiologically-active substances which are not normally present in the body, *i.e.*, drugs.

These substances must usually be applied in very small amounts. Their path can be followed by autoradiography[335]. In most cases, however, information about the chemical transformations of the labeled atoms will be needed. To facilitate analysis, inactive carrier can be added to the substance in the organ, or to the metabolite excreted. The yield obtained in the separation of the carrier agrees with that in the isolation of the radioactive drug, or of its metabolite (isotope dilution method). In choosing a carrier for a metabolic experiment, one's ideas about the nature of the metabolic process must serve as the guide.

The degradation product must, of course, be identified before quantitative determination. The following procedure may be used: a drug of high specific activity is administered to the animal, and the metabolic products are isolated as pure substances, the constitutions of which are unknown A series of parallel experiments are then performed with large amounts of the inactive drug, and in each experiment one of the pure, radioactive products is added to the mixture of the inactive metabolites. Only one of the products is thus labeled at a time, and each product can then be isolated individually in relatively large amounts from the mixture, and subjected to an analysis and to a determination of its structure. The advantage of this procedure is that the different radioactive materials are obtained individually before the separation of the main mass of metabolic products begins. This method of attack has been employed in the study of pentobarbital (nembutal)[336, 337].

Much progress has recently been made in research on tuberculosis[338]. Experiments with labeled isoniazide have been carried out on tuberculous guinea pigs (see[339] for the synthesis). The compound was taken up by many tissues, and disappeared again within a few days. The radiocarbon was stored in the region of the caseous material, but its chemical form was not determined[340]. Tuberculosis bacilli were also treated *in vitro* with radioactive isoniazide, and with other labeled drugs used in the treatment of tuberculosis[341, 342]. The brains of cats which had been treated with ^{14}C-isoniazide were examined by autoradiography[343]. Other experiments with labeled isoniazide have been performed on vitamin B_{12}-deficient rats[344].

Spinks has applied labeled vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) and the synthetic 'vitamin K_3' (2-methyl-1,4-naphthoquinone: 'menadione'). Both of these substances were administered orally to rats. Half of the K_1 and all of the K_3 was absorbed. When the bile was diverted by an external fistula, the absorption of K_1 decreased appreciably, while that of K_3 hardly changed. Both K_1 and K_3 are excreted in the urine; no radioactive carbon dioxide is expired. Deposition in the liver does not take place. It was found by paper chromatography and UV-spectroscopy that the excretion products of K_3 were the diglucuronide, the monosulfate, and the phosphate of the hydroquinone derived from the vitamin. A detailed theory of the mechanism of action of the vitamin has been based upon these experimental results[345–349].

Other radiocarbon experiments have been performed with the drugs pentobarbital[340, 350–352] (see above), benadryl[353], luminal[354] and methadone[355, 356]. The diethylamide of lysergic acid[357–359], serotonin[360] and nicotine[361] have also been studied. Work with labeled 2,4-dichlorphenoxyacetic acid[362–364] and other 'hormones'[365] are examples of investigations on plants. A review of the investigations on growth substances by means of isotopes is available[366]. The fates of ^{35}S-phenothiazine compounds have been followed[367, 368]. For the metabolism of ^{35}S-compounds, see also p. 142. As an example of work with bacteria, we may cite the investigation of the fate of sulfur-labeled sulfanilamide and related substances[369–371].

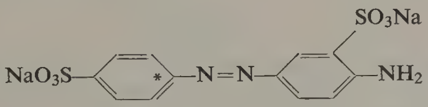
The metabolism of carcinogens and related substances has also been investigated with isotopes. A case is that of styrene labeled in the side group after subcutaneous injection into rats. The radiocarbon was distributed rapidly over the body. After injection 3 % of the radiocarbon were found unaltered in the expired air, 12 % appeared as $^{14}CO_2$, and the remainder was excreted in the urine in an unknown form[372]. Another group of workers investigated the fate of ^{14}C-urethane in normal and tumor mice; they were concerned mainly with the localization of the activity[373].

The fate of butter yellow (dimethylaminoazobenzene) has been studied more carefully. For the first experiments, this dye, which causes cancer of the liver, was labeled in the methyl group; it was found that most of the radiocarbon was exhaled as CO_2[374–376]. Other experiments have also shown that demethylation proceeds rapidly[377]. Subsequently, the dye was labeled in the carbon adjacent to the azo group in the ring which did not bear the dimethylamino group[378], and used for metabolic experiments on rats[379]. The carcinogen was administered as a single dose of 3 mg.

Some of the more important results are the following. (*a*) Radiocarbon is stored to a slight extent and for a few days only, and is rapidly excreted in the urine; this is true despite the fact that the cancer caused by butter yellow may appear a long time after ingestion—even after years[380]. (*b*) Though the liver does exhibit higher specific activity than any other organ, the difference from several other tissues (kidney and red blood cells) is not marked; indeed, the radiocarbon is stored longer in the erythrocytes than in the liver. The specificity of the carcinogenesis cannot, therefore, be ascribed to a concentration of the carcinogen by liver, but must be due to a particular susceptibility of the organ.

The objection may be raised that these conclusions are based on the determination of the fate of only one of the carbon atoms of butter yellow; in order to meet this criticism, further experiments were carried out in which the carbon atom adjacent to the dimethylamino group, or alternatively all the carbon atoms of both rings, were labeled. The results were always essentially the same as those obtained in the original experiment[381].

In connection with the butter yellow experiments, work was also done on the water-soluble sodium salt of 4-aminoazobenzene-3,4′-disulfonic acid; this compound was labeled in the position indicated with the asterisk [382, 383]. It was found that the distribution of radiocarbon among the organs of the rat was similar to that found with butter yellow, but that both the uptake and excretion were more rapid. In view of the relatively high activity of the liver in these experiments, it seems improbable that the carcinogenic action of butter yellow is merely a consequence of its good solubility in fatty matter; various other arguments can also be marshalled against a theory of this kind[377].



Another carcinogen, which attacks one particular organ, is β-naphthylamine; in this case it works on the gall bladder. Approximately 1 mg β-naphthylamine-8-^{14}C was administered intraperitoneally to rats[384]. The activity could be detected in the blood even after 10 weeks; after long periods only the erythrocytes remained active, as was the case with butter yellow. The excretion of radiocarbon occurred mainly in the urine; the respired air was inactive. 90–95 % of the radiocarbon were excreted within three days. Nothing is known about the chemical state of the carbon during its stay in the body. Analogous experiments with the same compound have been performed on rabbits[385].

Diethylstilbestrol labeled in the methylene group was fed to rats in 5 μg doses. After 6 hours, most of the radiocarbon was present in the intestinal contents and the feces; among the organs, the liver proved (weakly) active. The exhaled air was also inactive. The authors believe that absorption does not occur by way of the lymphatic system but rather by way of the portal vein. The fact that 96 % can be recovered from the bile of animals using bile fistulas was considered a proof of the enterohepatic circulation of this substance, The diethylstilbestrol excreted in the bile, presumably as a monoglucuronide, retains its full estrogenic activity[386]. Earlier investigations had led to the striking result that mammary gland, uterus, ovary and other reproductive organs do not accumulate radiocarbon from diethylstilbestrol[297] (also see p. 303). For work on other synthetic estrogens, the review literature should be consulted[300].

From an experimental standpoint, it is more difficult to work with carcinogens consisting of hydrocarbons with condensed ring systems, since these substances usually act after absorption through the skin; this however, is an ill-defined process. Yet on account of the great practical importance of these substances such investigations are of great significance. Here we can only refer to studies with labeled benzpyrene, dibenzanthracene, and methylcholanthrene[387–395]. Unfortunately, no results have yet been obtained which shed any light on the mechanism of carcinogenesis by these substances.

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Notes added in proof:

396 Considerable doubt has now been thrown on this assumption by W. Brzeski and W. Rücker (*Nature*, in press).

397 The biosynthesis of squalene and the sterols has now been discussed in an article on the history of the isoprene rule by L. Ruzicka (*Proc. Chem. Soc.*, (1959) 341).

APPENDIX

TABLE OF THE RADIOELEMENTS WHICH ARE MOST IMPORTANT IN PRACTICE, AND THEIR CHARACTERISTIC PROPERTIES

Table 13 presents a summary of the radioactive nuclei most suitable for biochemical applications. These nuclei are easy to prepare and have reasonable half-lives. The elements are arranged in order of increasing atomic numbers. Elements with little biochemical significance are not included; when several isotopes exist, only the more important ones are listed.

When a pile can be used to produce the radioelement, this is noted; other methods of preparation are given only when the pile cannot be used. The saturation activity (millicuries/g) is obtained after exposing the element with the naturally-occurring isotopic composition to a flux of thermal neutrons of 10^{11}cm^{-2}sec^{-1}. This saturation value is attained after irradiation for a time long compared to the half-life of the isotope.

Since the saturation activity is proportional to the neutron flux, the value obtained with any other neutron flux can be calculated immediately from the data given here. The impurities listed in column 10 are those which inevitably accompany the isotope from the given method of preparation.

More complete compilations of the known isotopes are available, *e.g.* O. STROMINGER, J. M. HOLLANDER and G. T. SEABORG, *Rev. Mod. Phys.*, 30 (1958) 585. Moreover, the International Atomic Energy Agency has issued, in 1959, an International Directory of Radioisotopes, in two volumes. In this Directory the prices of the radioelements, as quoted in the different countries, are listed. Volume 1 refers to "Unprocessed and Processed Radioisotope Preparations and Special Radiation Sources", Volume 2 to "Radioactive Chemical Compounds".

TABEL 13

RADIOELEMENTS WITH IMPORTANT BIOCHEMICAL APPLICATIONS

1 Atomic No.	2 Symbol	3 Isotope Mass Number	4 Isotope Half-life	5 Type of Decay	6 Energy of Particles (MeV)	7 Energy of γ-rays (MeV)	8 Principal Method of Preparation	9 Saturation Activity (mC/g)	10 Chief Impurities	11 Radioactive Daughters
1	H	3 ○	12.3 y	β^-	0.018		R Li (n, α)			
4	Be	7 ○	53 d	K, γ		0.48	B Li (d, n)			
6	C	14 ○	5570 y	β^-	0.155		R N (n, p)			
9	F	18 ○	112 min	β^+	0.65		R Li (n, t) He; O (t, n)			
11	Na	22 ○	2.6 y	β^+, K, γ	0.54	1.3	B Mg (d, α)			
11	Na	24	15.0 h	β^-, γ	1.39	2.76	R Na (n, γ)	32		
12	Mg	28	21.4 h	β^-	0.42	1.35	R ^{6}Li (n, t) He; ^{26}Mg (t, p)	0.03		^{28}Al
14	Si	31	2.6 h	β^-	1.47		R Si (n, γ)	0.26		
15	P	32	14.2 d	β^-	1.71		R P (n, γ)	12		
15	P	32 ○	14.2 d	β^-	1.71		R S (n̄, p)	~ 3000		
16	S	35	87 d	β^-	0.167		R S (n, γ)	0.54		
16	S	35 ○	87 d	β^-	0.167		R Cl (n, p)	~ $2 \cdot 10^5$		
17	Cl	36	$3.1 \cdot 10^5$ y	β^-	0.71		R Cl (n, γ)			
19	K	42	12.5 h	β^-, γ	3.6	1.53	R K (n, γ)	2.7		
20	Ca	45	153 d	β^-	0.25		R Ca (n, γ)	0.48		
20	Ca	45 ○	153 d	β^-	0.25		R Sc (n̄, p)			
23	V	48 ○	16 d	β^+, K, γ	0.7	1.31	B Ti (p, n)			
24	Cr	51	27.8 d	K, γ		0.32	R Cr (n, γ)	16		
25	Mn	54 ○	290 d	K, γ		0.84	B Cr (d, n)			
25	Mn	56	2.58 h	β^-, γ	2.8	2.1	R Mn (n, γ)	320		
26	Fe	55	2.94 y	K		0.006	R Fe (n, γ)	1.46	^{59}Fe	
26	Fe	55 ○	2.94 y	K		0.006	R Sz-Ch		^{59}Fe	
26	Fe	59	45 d	β^-, γ	0.46	1.3	R Fe (n, γ)	0.028	^{55}Fe	
26	Fe	59 ○	45 d	β^-, γ	0.46	1.3	R Sz-Ch	~ 100	^{55}Fe	
27	Co	60	5.25 y	β^-, γ	0.31	1.3	R Co (n, γ)	830		

30	Zn	65	245	d	β^+, K, γ	0 32	1.1	R Zn (n, γ)	6.8	69, 69*Zn	
33	As	76	26.5	h	β^-, γ	3.0	0.55	R As (n, γ)	90		
33	As	76 ○	26.5	h	β^-, γ	3.0	0.55	R Sz-Ch			
34	Se	75	125	d	K, γ		0.4	R Se (n, γ)	4.9		
35	Br	82	36	h	β^-, γ	0.44	1.48	R Br (n, γ)	22		
38	Sr	89	51	d	β^-	1.46		R Sr (n, γ)	0.078		
38	Sr	89 ○	51	d	β^-	1.46		R U (n, f)		^{90}Sr	^{90}Y
38	Sr	90 ○	28	y	β^-	0.54		R U (n, f)		^{89}Sr	^{90}Y
42	Mo	99	67	h	β^-, γ	1.23	0.78	R Mo (n, γ)	1.7		
53	I	131 ○	8.0	d	β^-, γ	0.61	0.64	R Te (n, γ) $^{131}_{52}\mathrm{Te}\xrightarrow{\beta}$ R U (n, f)			
53	I	132 ○	2.3	h	β^-, γ	2.2	0.69	R Te (n, γ) $^{132}_{52}\mathrm{Te}\xrightarrow{\beta}$			
56	Ba	131	11.5	d	K, γ		0.5	R Ba (n, γ)	0.03		^{140}La
56	Ba	140 ○	12.8	d	β^-, γ	1.02	0.5	R U (n, f)			
79	Au	198	2.69	d	β^-, γ	0.96	0.41	R Au (n, γ)	780		
80	Hg	203	47	d	β^-, γ	0.21	0.28	R Hg (n, γ)	5.8	^{197}Hg	
82	Pb	210 (RaD) ○	19.4	y	β^-, γ	0.063	0.05	N			RaE, RaF
82	Pb	212 (ThB) ○	10.6	h	β^-, γ	0.57	0.24	N			ThC, ThC′ ThC″
83	Bi	210 (RaE) ○	5.0	d	β^-	1.17		N			RaF
83	Bi	210 (RaE)	5.0	d	β^-	1.17		R Bi (n, γ)	0.12		RaF
83	Bi	212 (ThC) ○	1	h	α, β^-, γ	2.2 (β)	1.81	N			ThC′, ThC″
84	Po	210 (RaF) ○	138	d	α	5.3		N R Bi (n. γ) $^{210}_{83}\mathrm{Bi}\xrightarrow{\beta}$			
90	Th	234 (UX 1) ○	24	d	β^-, γ	0.2	0.09	N			UX 2[$\tau = 1.14$ min, E (β^-) = 2.3]
92	U	234 (U II) ○	$2.5 \cdot 10^5$	y	α, γ	4.76	0.05	N			

Explanation of symbols:

*	excited state	N	naturally radioactive element
○	carrier-free or carrier-poor	R	pile
f	fission	B	accelerator
Sz-Ch	Szilard-Chalmers effect	$\bar{n}$	fast neutrons

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